REGULATION OF 2-5A DEPENDENT RNase AT THE LEVEL OF ITS PHOSPHORYLATION

1991

SUKUMAR

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

APPROVAL SHEET

RADUATE EDUCATION

TEACHING HOSPITALS
WALTER REED ARMY MEDICAL CENTER
NAVAL HOSPITAL, BETHESDA
MALCOLM GROW AIR FORCE MEDICAL CENTER
WILFORD HALL AIR FORCE MEDICAL CENTER

Title of Dissertation: "Regulation of 2-5 A Dependent RNase at the Level of its Phosphorylation"

Name of Candidate: Niru Sukumar
Doctor of Philosophy Degree
June 26, 1991

Dissertation and Abstract Approved:

Committee Chairperson	6/26/91 Date
Committee Member	6/26/91 Date
Committee Member	6/26/91 Date
Committee Member	6/26/91 Date
Committee Member	9/4/91 Date

The author hereby certifies that the use of any copyrighted material in the thesis manuscript entitled:

"Regulation of 2-5 A Dependent RNase at the Level of its Phosphorylation"

beyond brief excerpts is with the permission of the copyright owner, and will save and hold harmless the Uniformed Services University of the Health Sciences from any damage which may arise from such copyright violations.

Niru Sukumar
Department of Pathology
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Dissertation

Regulation of 2-5A dependent RNase at the level of its phosphorylation

Niru Anita. Sukumar

Candidate, Doctor of philosophy, 1991

Dissertation directed by:

Robert H. Silverman

Professor, Department of Pathology

The 2-5A system is an interferon-regulated, antiviral pathway which results in the degradation of viral RNA and may also be involved in cell growth control. Interferon treatment of cells induces 2-5A synthetases which, upon activation by double stranded RNA, produce 2',5'-linked oligoadenylates (2-5A). The only well-characterized, biochemical effect of 2-5A is its activation of 2-5A dependent RNase, an interferon-inducible 80 kDa protein that cleaves single stranded RNA. Here I describe a novel level of regulation in the 2-5A pathway.

Since 2-5A dependent RNase has been implicated in

growth control, my objective was to determine, how activators of protein kinase C, which in several cell lines stimulate growth or inhibit differentiation would lead to qualitative as well as quantitative changes in the pattern of 2-5A dependent RNase.

Murine JLS-V9R cells were treated with interferon in the presence or absence of activators or an inhibitor of protein kinase C or a phosphatase inhibitor. Interestingly, the protein kinase C activators and the phosphatase inhibitor down-regulated the interferon-induction of 2-5A dependent RNase, whereas, the protein kinase inhibitor superinduced 2-5A dependent RNase as measured by its ability to covalently bind $p(A2'p)_2(br^8A2'p)_2A3'-[^{32}p]Cp$ (subsequently the "2-5A probe"). Assays for 2-5A in cell extracts indicated that these results were not due to competition between endogenous 2-5A and the 2-5A probe for binding. Instead, the observed regulation appeared to be due to modulation of its state of phosphorylation. Accordingly, 2-5A dependent RNase was shown to be a substrate for protein kinase C <u>in vitro</u>.

To extend these findings, subsequent studies were performed with recombinant 2-5A dependent RNase. Phosphorylation of the recombinant 2-5A dependent RNase with unlabeled ATP and protein kinase C, inhibited its binding to

the 2-5A probe. Similarly, binding of partially purified 2-5A dependent RNase to unlabeled 2-5A, inhibited its subsequent phosphorylation. These findings suggest that the phosphorylation site in 2-5A dependent RNase may be included in its 2-5A binding domain.

It will be of interest to determine if a phosphorylation/dephosphorylation mechanism could regulate the 2-5A dependent RNase in a cell cycle dependent manner and whether its functions and regulatory properties overlap with those of other growth suppressor genes.

REGULATION OF 2-5A DEPENDENT RNase AT THE LEVEL OF ITS PHOSPHORYLATION

by

Niru Anita Sukumar

Dissertation submitted to the
Faculty of the Department of Pathology
Graduate Program of the
Uniformed Services University of the Health Sciences
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1991

DEDICATION

To Rahul and Shaan

ACKNOWLEDGEMENT

I would like to thank the members of the Department of Pathology and my committee for their encourgement and help during the preparation of this dissertation. I would especialy like to thank my advisor, Dr Robert Silverman for his guidance in completing this research. I also thank Drs. Kenneth Minton, Clifford Snapper, and Philip Grimley. A very special thanks to Dr. James Mond; without his encouragement, friendship and guidance I may not have completed my Ph.D. My committee's standards as scientists have become the guidelines for my future.

Thanks to Zhou Amin, Bret Hassel, Dibu Sengupta and Ratan Maitra. I valued both the scientific and technical knowledge I gained from them, and their great company in the lab. They made graduate school a lot of fun along the way. I'd almost do it again.

Last, but not least, thanks to my family who have endured, for their love, patience, and understanding; they now deserve my full attention.

Table of Contents

List of Figures

List of Tables

List of Abbreviation

ATP	adenosine triphosphate
BSA	bovine serum albumin
CMF-PBS	calcium and magnesium free phosphate buffered saline
cpm	counts per minute
DAG	diacylglycerol
dbcAMP	Dibutyryl cAMP
dsRNA	double stranded RNA
DiC8	1,2-dioctanoyl-sn-glycerol
EC	Embryonal Carcinoma
EMEM	Earl's modified Eagles medium
EtOH	ethanol
FBS	fetal bovine serum
HPLC	high performance liquid chromatography
IBMX	3-Isobutyl-1-methylxanthine
IFN	Interferon
IND	Indolactam
H-7	1[(5-isoquinolinylsulfonyl)]-2-methylpiperazine dihydrochloride
MeOH	methanol
NP40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PKC	protein kinase C

PKA	protein kinase A(cAMP dependent protein kinase)
PI	phosphatidylinositol
PS	phosphatidylserine
Ptdlns(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
SDS	sodium dodecyl sulphate
TCA	trichloro acetic acid
TPA	12-O-tetradecanoylphorbol-13-acetate
ug	microgram
ul	microliter
UTR	untranslated region
2-5A-dependent RNase	2-5A-depRNase
2-5A	$p_x(A2'p)_nA$; x = 2 or 3, $n \geq 2$
Br-2-5A[^{32}p]Cp	$p(A2'p)_2(br^8A2'p)_2A3[^{32}p]Cp$

INTRODUCTION

Interferons (IFNs) are a family of proteins grouped into three major classes based on their biological, immunological and chemical properties. A wide range of biological activities are observed in IFN-treated cells, including an antiviral effect (Nilsen et al.,1981; 1982), inhibition of cell growth (Knight, 1976; Taylor-Papadimitriou, 1979), modulation of the immune response (Zarling et al., 1979), cell membrane modifications (Knight and Korant,1977), anti-tumor activity (Gresser and Tovey, 1978) and regulation of differentiation (Keay and Grossberg, 1980; Rossi et al., 1977; Maor et al., 1990). Furthermore, IFN has been shown to be produced and secreted by cells in an autocrine fashion during their differentiation process (Maor et al., 1990). The presence of IFN in several organs of normal individuals suggests that it is important in normal physiology (Tovey et al., 1987), especially in maintaining pregnancy (Roberts, 1989).

Three types of IFNs, alpha, beta, and gamma, and the genes encoding them have been identified and extensively characterized (Nagata et al., 1980; Derynck et al., 1980). IFN actions are mediated through interactions with receptors present at the cell surface (Aguet, 1980; Mongensen and

Bandu, 1983; Hannigan and Williams, 1986). Competitive binding of human IFNs on human cells, seem to indicate that all human IFNs-α and β have a common receptor, while IFN gamma is recognized by a distinct receptor system (Aguet et al., 1984; Merlin et al., 1985). Affinity labeling using iodinated IFN-α and cross-linking agents or ligand blotting showed that IFN-α could be cross-linked with a 95-100 kDa component present in the plasma membrane (Vanden Broecke and Pfeffer, 1988; Eid et al., 1988; Uze et al., 1988; Schwabe et al., 1988). The abundance of IFN receptors on the cell surface do not reflect the sensitivity of cells to IFN. In fact, IFN receptors are present in all tissues and even on the surface of most IFN-resistant cells (Aguet and Mogensen, 1983). While the genes for IFN-α and β are located on chromosome 9 (Owerbach et al., 1981) and that for gamma on chromosome 12 (Pestka et al., 1987); the genes for the IFN receptor proteins which are not linked to the structural genes for human interferons-α and -β, are located on chromosome 21 (Gilles et al., 1990), or the gene for IFN gamma receptor, which is on chromosome 6 (Aguet et al., 1988; Rashidbaigi et al., 1986).

When an IFN molecule binds to its cell surface receptor a series of poorly understood events is set in motion, culminating in the induction, or inhibition of a number of cellular proteins. In spite of much progress, basic

questions concerning the interferon system remain unanswered. For instance, how IFN-IFN receptor complexes function? What are the roles of the various IFN induced, dsRNA-activatable enzymes (i.e. 2' 5' oligoadenylate synthetases and the dsRNA-dependent protein kinase) during cellular proliferation and cell growth arrest?

IFN that has been purified to homogeneity has been shown to inhibit the proliferation of many cell types and to slow the growth of certain tumors introduced experimentally into animals (Gresser & Tovey, 1978). However, the mechanisms by which IFNs inhibit cell proliferation are largely unknown.

Other effects of IFNs on cells include, an alteration in the methylation of viral (and perhaps cellular) mRNAs (Sen et al., 1975; Kahana et al., 1981) and a decrease in the proportion of unsaturated fatty acids in membrane phospholipids, which results in an increase in the rigidity of the cell membrane (Chandrabose et al., 1981; Apostolov et al., 1981). While the biochemical basis for all of these cellular responses is not well understood, certain enzymatic pathways thought to play a role in the action of IFNs have been investigated.

THE 2-5A SYSTEM

Since IFNs are pleiotyic mediators of such important cellular responses as cell growth inhibition and immunoregulation, it is tempting to speculate that the $P_x(A2'p)_nA$; x=2 or 3, n ≥ 2 (2-5A) system may be involved in the control of these processes.

The 2-5A system involves three types of enzymes:

1. The 2-5A synthetase, which synthesizes 2-5A.
2. The 2-5A-dependent RNase (2-5A-depRNase).
3. A 2'5'-phosphodiesterase which degrades 2-5A

Interferon treatment of most animal cells induces an enzyme, the 2-5A synthetase, that on activation by double-stranded (ds) RNA converts ATP to (2-5A) (Hovanessian et al., 1977) [Fig. 1]. 2-5A at subnanomolar levels activates an endoribonuclease, 2-5A-depRNase (Slattery et al., 1979) resulting in the cleavage of single-stranded RNA on the 3' side of UpNp sequences (Wreschner et al., 1981; Floyd-Smith et al., 1981). The "2-5A system" is completed by a third enzyme, a 2',5'-phosphodiesterase (2',5'-PDi) which degrades $ppp(A2'5')_nA$ to AMP and ATP (Minks et al., 1979) [Fig. 2]. Thus in the absence of continuing synthesis of 2-5A, the activation of the 2-5A-depRNase is transient. This

The Trimer Species of 2-5A

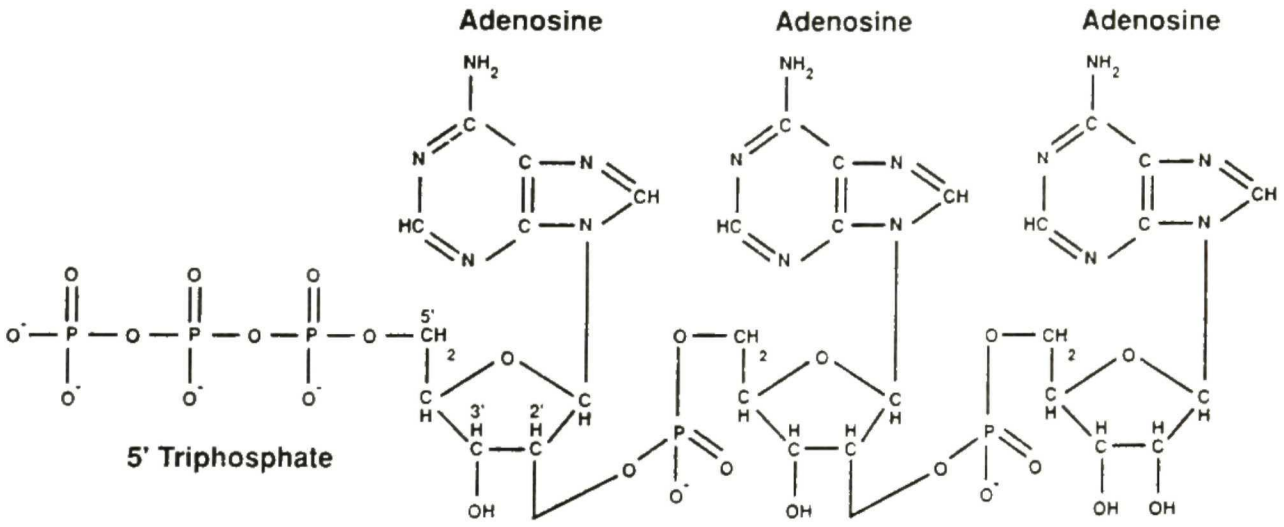

Figure 1.

Struture of 2-5A Trimer Triphosphate

5'-O- TRIPHOSPHORYLADENYLYL - [2'->5']

ADENYLYL-[2'->5']ADENOSINE. "2-5A"

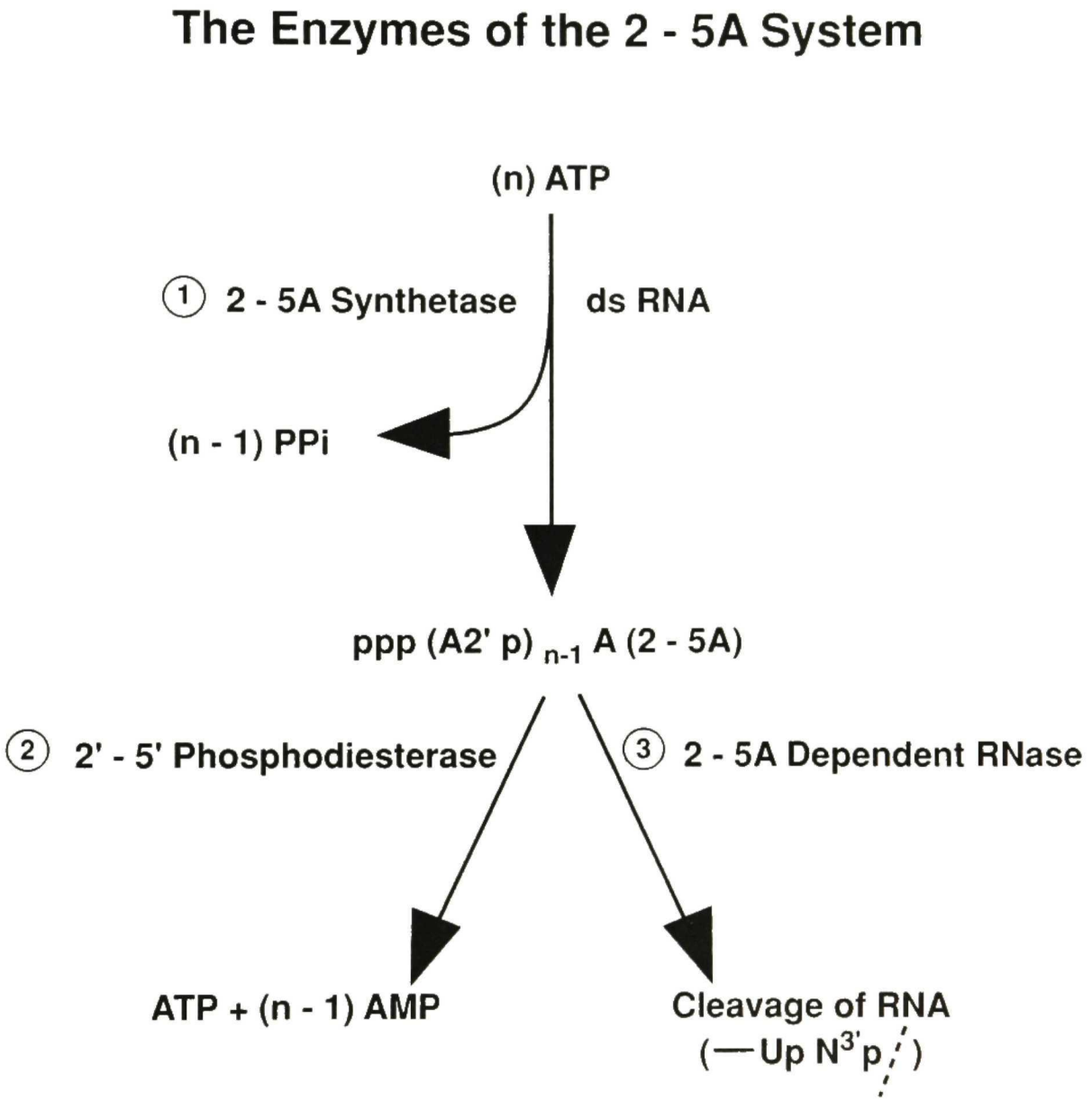

Figure 2.

THE "2-5A" PATHWAY

pathway results in inhibition of protein synthesis in both cell-free systems and intact cells (Williams and Kerr, 1979; Williams et al., 1978; Baglioni et al., 1978). Activation of the 2-5A-depRNase is the only function of 2-5A which has been clearly established. However, there are indications that 2-5A may function outside the context of the IFN response and that these compounds may participate in control of processes other than RNA degradation (Reid et al, 1983; Krishnan et al, 1981).

2-5A Synthetase.

The intracellular activity of IFN is mediated by a variety of IFN-induced proteins, of which the 2-5A synthetase has been one of the best characterised (Chebath et al., 1987). In the presence of Mg^{++} and dsRNA, 2-5A synthetase polymerizes ATP into 2' 5' linked oligoadenylates.

The reaction catalysed by 2-5A synthetase is

$$(n+1)\ ATP \xrightarrow[dsRNA]{} (2',5')pppA(pA)_n + nPPi;\ (n=1 \text{ to } 15)$$

The predominant products of the reaction are dimers, trimers

and tetramers possessing a 5' triphosphate; these oligomers are characterized by their sensitivity to digestion to snake venom phosphodiesterase and resistance to RNase T_2, P_1, and U_2, micrococcal nuclease, spleen phosphodiesterase and pancreatic RNase (Johnston, M.I. and Torrence, P. 1984).

Four forms of 2-5A synthetase have been identified in humans using polyclonal antibodies to a peptide common to all forms of the enzyme (Chebath et al., 1987a). Enzymes of 40 and 46 kDa forms of 2-5A synthetase are coded by 1.6-and 1.8-Kb mRNAs, derived from a single 14 Kb gene by differential 3' splicing (Benech et al., 1985, Saunders et al., 1985). They are identical in their first 346 amino acid residues, but are different at their C-terminal ends (Benech et al., 1985). Microsome associated, 100 kDa, and cell membrane associated, 69 kDa, forms of 2-5A synthetase are probably encoded by a different gene or genes, not yet identified (Hovanessian et al., 1987 and 1988). Human and murine 2-5A synthetase genes display significant homology in the promoter region (Cohen et al., 1988) as well as in the coding regions (Saunders et al., 1985; Ichii et al., 1986). In the mouse system, a 4.0 Kb mRNA coding a 100 KDa cytosolic 2-5A synthetase and a 1.6 Kb mRNA coding a 30 KDa nuclear enzyme are thought to correspond to the 1.8 and 1.6 Kb human mRNAs (St. Laurent et al., 1983). An additional 71 KDa enzyme has been identified in immunoblots of mouse cell

proteins (Chitayat et al., 1987). Two non-allelic 2-5A synthetase genes have been cloned in mice, and Southern blots of genomic DNA suggests the existance of additional genes (Cohen et al., 1988).

Human chromosomes 11 and 12 have been suggested to encode 2-5A synthetases (Shulman et al., 1984; Williams et al., 1986). The small size 2-5A synthetases (40-and 46-kDa) have been assigned to chromosome 12 (Williams et al., 1986) whereas the evidence for chromosome 11 has been obtained using an enzyme activity assay (Shulman et al., 1984). It is possible that chromosome 11 might encode either or both of the 69- and 100- kDa enzymes. Cell-specific expression of the various 2-5A synthetase forms has been shown, suggesting differences in their regulation and their involvement in IFN action (Chebath et al., 1987a). The purification of these enzyme forms has revealed distinct structure and concentration requirements of dsRNA for maximal activation (Chebath et al., 1987a; Hovanessian et al., 1988).

2-5A-depRNase

One of the IFN-induced proteins whose level is higher in differentiated embryonal carcinoma (EC) cells (Krause et al., 1985), as opposed to undifferentiated EC cells, in

Physiological States in which 2-5A-Dependent RNase is Induced

1. Cell Growth Arrest
   - Confluency
2. Cellular Differentiation
3. Interferon Treatment
4. Serum Starvation

Figure 3.

Physiological states in which

2-5A-depRNase is induced

serum starved (Floyd-smith et al., 1988), and confluent JLS-V9R cells (Jacobsen et al., 1983), is the 2-5A-depRNase (Fig. 3). In various murine cell lines, substantial fluctuations in levels of the 2-5A-depRNase were observed as a function of interferon treatment, growth rate and cell differentiation. Furthermore, the levels of 2-5A synthetase activity also vary considerably in different cell lines and tissues as a function of growth rate, differentiation, and hormonal response (Etienne-Smekens et al., 1983; Friedman-Einat et al., 1982; Krishnan, I.and Baglioni,C., 1981; Stark et al., 1979). Interferon treatment of Daudi and Hela (Silverman et al., 1982a and 1982b) or mouse L (Cayley et al., 1982) cells resulted in an increase (about 2-fold) in levels of the 2-5A-depRNase. Murine JLS-V9R cells which contain low basal levels of 2-5A-depRNase develop a 10-to 20-fold increase after interferon treatment (Jacobsen et al., 1983). Regulation of 2-5A-depRNase is not, however, limited to interferon treatment. In JLS-V9R cells, a 6-8-fold increase in 2-5A-depRNase was observed during the transition from the subconfluent, actively growing state to the confluent stationary phase (Jacobsen et al., 1983). The increase in 2-5A-depRNase during confluency was not due to the production of IFN by the cells. The induction of 2-5A-depRNase during confluency preceded an inhibition of [^{3}H]thymidine incorporation into DNA by the cells (Jacobsen et al., 1983). This suggested that the 2-5A pathway may be

involved in growth control in these cells. Also, intracellular levels of 2-5A synthetase, vary in inverse relation to mitogenic activity, suggesting that the 2-5A system may be involved in the control of cell growth (Kimchi et al., 1981a and 1981b). As regulators of cell growth and differentiation, the IFN inducible enzymes of the 2-5A system could act by inhibiting cell growth and promoting differentiation, reversing transformed phenotype, or inhibiting the functions of oncogene products.

2-5A depRNase has been purified to homogeniety by electroelution from a polyacrylamide gel (Silverman et al., 1988). Scatchard plot analysis, using mouse liver homogenate showed a single noninteracting 2-5A binding site with a K_a of 2.5×10^{10} M^{-1} (Silverman et al., 1988).

2'5'-phosphodiesterase.

The third enzyme of the 2-5A pathway, referred to as 2'5' phosphodiesterase (2'5'PDi), produces 5'-AMP (from internal and 2'-terminal adenosines) and ATP from the 5' terminus of 2-5A (Minks et al., 1979). It is ubiquitous in mammalian cells and requires Mg^{2+} for activity (Torrence, P.F., Imai, J. and Johnston, M.I.,1983). Its activity has been shown to vary with changes in growth rate. Confluent

cells have been shown to have one-third the 2'5'PDi activity and almost twice the synthetase activity as do growing, mitogenically or serum stimulated cells, resulting in a 5-fold increase in the synthesis to degradation ratio (Kimchi et al., 1981). A dual action of 2'5'PDi and synthetase has been implicated in providing the cell with a rapid response mechanism to molecular modulators (Johnston, M.I. and Torrence, P.F., 1984).

A regulated activation of the 2-5A-depRNase, thus protecting the cell from indiscriminate RNA degradation, was originally conceived as the role of 2'5'PDi. However, purification of 2'5'PDi from bovine spleen, showed that the Km of this enzyme for 2-5A was in the uM range, far above the nM concentrations known to be biologically active (Johnston and Hearl, 1987). This suggested that the 2'5'PDi may be ineffective in directly limiting 2-5A-depRNase activation.

Inactivation of the biological activity of 2-5A in vivo could result from cleavage of the phosphodiester bonds. It may also become inactive by removal of the 5' beta and gamma phosphoryls by phosphatases, since these moieties are required for nuclease activation and subsequent inhibition of protein synthesis. The finding of core and monophosphate 2-5A in several tissues and cell types (Reid et al., 1985;

Laurence et al., 1984) suggested that 2-5A is initially inactivated by removal of one or more of the 5' phosphoryl groups by nonspecific cellular phosphatases. The complete breakdown of 2-5A is likely to be of biological significance as high concentrations have been reported to be cytotoxic (Johnston and Torrence, 1984); furthermore, this activity may be important in the recovery of IFN treated cells from viral infection (Johnston and Hearl, 1987). The enzyme 2'5'PDi may also be involved in the inhibition of protein synthesis by cleaving the CCA terminus of tRNA resulting in a reversible inactivation of the tRNA (Schmidt et al., 1979).

2-5A-depRNase and cell growth: Wider significance of the 2-5A system.

The enzymes of the 2-5A system are widely distributed in a variety of reptilian, avian and mammalian cells and tissues (Nilsen et al., 1981; Cayley et al., 1982) and levels of 2-5A synthetase and 2-5A-depRNase can vary with growth, differentiation and hormone status, suggesting a broader role beside its antiviral functions, in normal cell growth or function.

Antagonistic effects between IFN and various cell

growth factors, suggested that cellular proliferation may be regulated by opposing actions of growth-promoting factors and IFNs (Inglot et al., 1983). The expression of the oncogene product (pp60 src), which regulates growth of transformed cells and is a substrate for PKC, is suppressed by IFNs (Lin et al., 1983). Taken together, these findings favored the possibility of an interaction between PKC and the IFN induced enzymes during growth regulation. In growth arrested cells, the role of 2-5A-depRNase may be to control or modulate the expression of oncogenes governing abnormal proliferation.

Regulation of 2-5A-depRNase: A role for Protein Kinase C.

A possible role for an interaction between PKC and the 2-5A-depRNase in growth control was supported by two lines of evidence; (1) Studies indicating that cell growth arrest could be controlled or influenced by some specific negative growth factor(s) enhanced during differentiation, such as the enzymes 2-5A synthetase and 2-5A-depRNase (Clemens et al., 1985; Krause et al., 1985) and (2) that retinoids which induce cellular differentiation and subsequently enhance the level of 2-5A-depRNase, may act as antagonists to PKC (Cope et al., 1984).

Protein Kinase C and Control of Cellular Processes by Phosphorylation

EXTRACELLULAR SIGNAL (1st Messenger)

1. Cell Surface Antigens
2. Growth Factors
3. Hormones
4. Tumor Promoters

↓

membrane

Stimulates Inositol Phospholipid Breakdown (2nd Messenger)

+ Diacylglycerol + Ca^{2+} (from Ca^{2+} mobilization within the cell)

↓

MODULATORS

- Protein Kinase C
- Phosphatases

↓

TARGETS

- Other Kinases
- Regulatory Enzymes
- Structural, Cytoskeletal Proteins

↓

RESPONSE

- Physiological or Metabolic Functions

Figure 4.

Protein Kinase C and control of cellular processes by phosphorylation

B. Protein Kinase C

The process of cell differentiation is characterized by a gradual limitation in the capacity of cells to proliferate. A universal aspect of cellular growth control mechanisms is that they must ultimately respond to extracellular signals (Fig. 4).

The enzyme PKC, was first found in 1977 as a proteolytically activated protein kinase in many tissues, with brain having the highest activity (Kuo et al., 1980). It was shown to be a Ca^{2+} activated, phospholipid-dependent enzyme and was firmly linked to signal transduction by the demonstration that diacylglycerol, one of the earliest products of signal- induced inositol phospholipid breakdown, greatly increased the affinity of PKC for Ca^{2+}, thereby activating it (Takai et al., 1979). Although Ca^{2+} and diacylglycerol signals are transient, the two pathways are essential and often synergistic in evoking subsequent cellular responses (Kikkawa and Nishizuka 1986). PKC has a crucial role in signal transduction for a variety of biologically active substances which activate cellular functions and proliferation (Nishizuka, 1984).

PATHWAYS OF SIGNAL TRANSDUCTION: Role of PKC in cell surface signalling and tumor promotion.

PKC is thought to play a central role in the regulation of a multitude of cellular functions through its activation by growth factors and other agonists such a phorbol esters. These functions include cell growth and proliferation (Rosengurt et al., 1984; Kaibuchi et al., 1985; Person et al., 1988), control of ion transport channels (Madison et al., 1986; Farley and Auerbach, 1986), and release of various hormones (Negro-Vilar and Lapetina, 1985). The findings that PKC is the intracellular receptor for phorbol esters, which are tumor promoters that mimic the activating effect of DAG and induce the transcription of a wide array of proto-oncogenes such as, c-myc, c-fos and c-sis, favored the hypothesis that a perturbed activation of PKC may result in tumor promotion (Kelly et al., 1983; Coughlin et al., 1985; Greenberg and Ziff, 1984; Kruijer et al., 1984; Colamonici et al., 1986).

PKC is widely distributed in tissues and organs of mammals and other organisms (Kuo et al., 1980). PKC isotypes consist of a single polypeptide chain (molecular weight 77,000-80,000 dalton) that appears to be composed of two functionally different domains which can be separated with Ca^{2+}-dependent thiol proteases. One is a hydrophobic domain

that may bind to membranes; the other is a hydrophylic domain that carries the catalytically active center. The hydrophylic fragment is fully active without Ca^{2+}, phospholipid and diacylglycerol. A class of Ca^{2+}-dependent thiol proteases which are active at the 1-10 uM range of Ca^{2+} preferentially cleave the activated form of PKC irreversibly (Huberman et al., 1979). The response of inositol phospholipids to the activation of cell surface receptors was first recognized by Hokin and Hokin, (1953) who showed using pancreatic slices and later with other cell types that breakdown and resynthesis of inositol phospholipids occur in response to a wide variety of external signals, and this phospholipid breakdown may open a Ca^{2+} gate (Hawthorne., 1982; Irvin et al., 1979). Inositol phospholipids most frequently contain the 1-stearoyl-2-arachidonyl-glycerol backbone, and a small portion of PI(phophatidylinositol 4-phosphate) contains an additional phosphate at position 4 (Phosphatidylinositol 4-phosphate, PIP) or two phosphates at positions 4 and 5 (PIP2) of the inositol moiety. These minor phospholipids, are produced through sequential phosphorylation of PI. In various tissues, signal-induced breakdown of PIP2 appears to precede the previously known PI response, and PIP2 has recently been regarded as a prime breakdown target (Fisher et al., 1984). In many tissues PI and PIP also disappear when cell surface receptors are stimulated, and it is not absolutely clear

whether they are hydrolyzed directly or are phosphorylated to form PIP2 before being hydrolyzed (Kikkawa and Nishizuka., 1986). Plausible evidence at present suggests that in many cell types, such as platelets, the three inositol phospholipids are broken down, probably at different rates, starting at different times, and resulting in the formation of diacylglycerol and inositol mono-, bis, and trisphosphate (Fisher et al., 1984). Diacylglycerol is only transiently produced in membranes, presumably due both to its conversion back to inositol phospholipids and to its further degradation to arachadonic acid for thromboxane and prostagladin synthesis. Thus, the information of external signals may flow from the cell surface into the cell interior through two pathways, Ca^{+2} mobilization and PKC activation.

PKC is a family of enzymes consisting of at least seven isotypes (α,β_1,β_{11},gamma,δ,ϵ,and zeta), which exhibit distinct tissue-specific patterns of expression (Kikkawa et al., 1989). These are amphitropic proteins, that have been shown to undergo complex posttranslational regulation, translocating between membrane and cytoplasm according to the local concentrations of calcium, phospholipids, and diacylglycerols, and it is thought that the enzymes are physiologically active only in the membrane associated state (Ashendel et al., 1985; Burn 1988; May et al., 1985). Acute

and transient translocation of PKC from cytoplasmic to the membrane compartment can be induced with appropriate stimuli in different experimental systems.

Growth-controlling factors.

A well known inhibitor of growth is IFN; others include chalones (Iverson et al., 1981), and proteins isolated from density-inhibited BSC-1 cells and 3T3 cells(Whittenberger et al., 1978; Liberman, et al., 1981; Datta and Natraj., 1990). A variety of agents capable of promoting growth have also been described. Several chemically diverse agents such as polypeptide growth factors, cyclic nucleotides, corticosteroids, cholera toxin, tumor promoters, and prostaglandins, acting singly or in combination, can inhibit or initiate DNA synthesis in cell cultures. One class of naturally occurring products, the phorbol ester tumor promoters, are potent modulators of growth and cellular differentiation, either inhibiting(Yamasaki et al., 1977; Miao et al., 1978) or inducing (Huberman et al., 1979; Kikkawa et al., 1986) differentiation, depending on the target cell. In the present study I thus investigated the effects of the tumor promoter TPA and related compounds on the JLS-V9R cells, in an attempt to gain insight into the role of the 2-5A system in cellular growth and

differentiation.

Protein Kinase C activators and inhibitors.

Mechanism of action.

Tumor promoters constitute a family of compounds which, although not carcinogenic by themselves, drastically increase the incidence of tumors when applied repeatedly to animals that have received a sub-threshold dose of a carcinogen (Hecker, E. 1978). Their mechanism of action is of considerable interest, with attention focusing on their biological effects in various types of cultured cells.

Tumor promoting phorbol esters, such as TPA, have a molecular structure that in part is very similar to that of diacylglycerol (Fig. 5) and activate PKC directly both in vitro and vivo (Castagna et al., 1982). There is ample evidence to suggest that PKC is probably a prime target of tumor promoters (Castagna et al., 1984; Yamanishi et al., 1983). Agents such as Mezerein (Couturier et al., 1984) and Indolactam (Fig. 5) that are structurally unrelated to phorbol esters also activate PKC, suggesting that a diacylglycerol-like structure is not always essential and that many tumor promoters so far identified may induce a membrane perturbation analogous to that caused by

diacylglycerol

Indolactam:

Indolactam, a derivative of indole, is a potent activator of PKC, and binds to the same site on the enzyme at which the phorbol esters act (Sugimura et al., 1982). It consists of an indole nucleus carrying an additional nine-membered lactam ring (Cardellina et al., 1979).

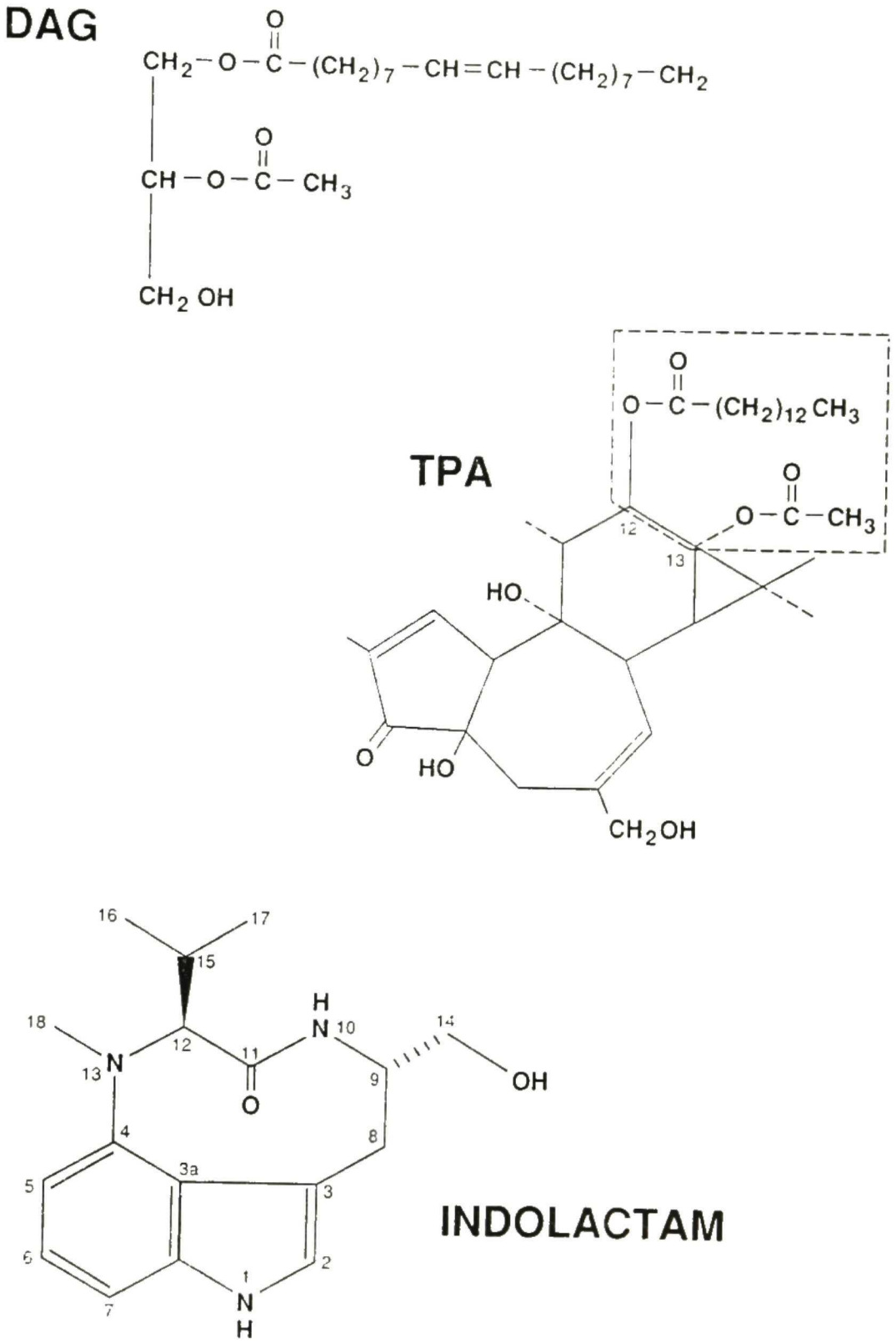

Figure 5.

Chemical Structure of Protein Kinase C Activators

H-7:

1-(5-isoquinolinylsulfonyl)-2-methylpiperazine(H-7) is a potent inhibitor of kinases; inhibiting PKC with greater propensity than other kinases (Fig. 6). It competes with ATP for free enzyme, but does not interact with the same enzyme form as does the phosphate acceptor (i.e., enzyme-ATP complex){Hidaka et al 1984}. This compound serves as a useful pharmacological tool for elucidating the biological significance of protein kinase mediated reactions.

Okadaic acid:

Okadaic acid is a tumor promoter and a potent and specific inhibitor of protein phosphatases 1 and 2A. It is a polyether fatty acid first isolated from the marine sponges Halichondria okadadaii and Halichondoria melanodocia (Tachibana et al., 1981) [Fig. 6]. The first clue to its mechanism of action came with the discovery that okadaic acid caused a long lasting contraction of isolated, vascular smooth muscles from human umbilical arteries (0.1-100 uM) and rabbit aorta (10 uM) (Shibata et al.,1982). Smooth muscle contraction is triggered by the phosphorylation of one of the small subunits of myosin, the P-light chain, suggesting that okadaic acid might activate myosin P-light chain kinase or inhibit a myosin P-light chain phosphatase.

Takai and co-workers established that the latter idea was correct and went on to show that okadaic acid is a very potent inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). PP1 and PP2A are two of the four major protein phosphatases in the cytosol of mammalian cells that dephosphorylate serine and threonine residues (Cohen., 1989).

Okadaic acid, being hydrophobic, can enter cells and studies with ^{32}P-labelled hepatocytes, adipocytes and chromofin cells (Haystead et al., 1989; Haavik et al., 1989) showed that addition of okadaic acid to the incubation medium increased the phosphorylation states of many proteins within minutes, and stimulated overall levels of protein phosphorylation by 2.5 to 3 fold. Okadaic acid had no effect on ATP levels and no unexpected toxic effects in short term incubations. Proteins whose phosphorylation was increased in hepatocytes and adipocytes included the rate-limiting enzymes of glycogen and lipid metabolism, glycolysis and gluconeogenesis(Haystead et al., 1989). An increasing number of studies using okadaic acid have revealed novel processes that are controlled by phosphorylation/dephosphorylation mechanisms. It mimics the dramatic stimulation of glucose transport into adipocytes by insulin, suggesting that this process is mediated by phosphorylation of a protein on serine/threonine

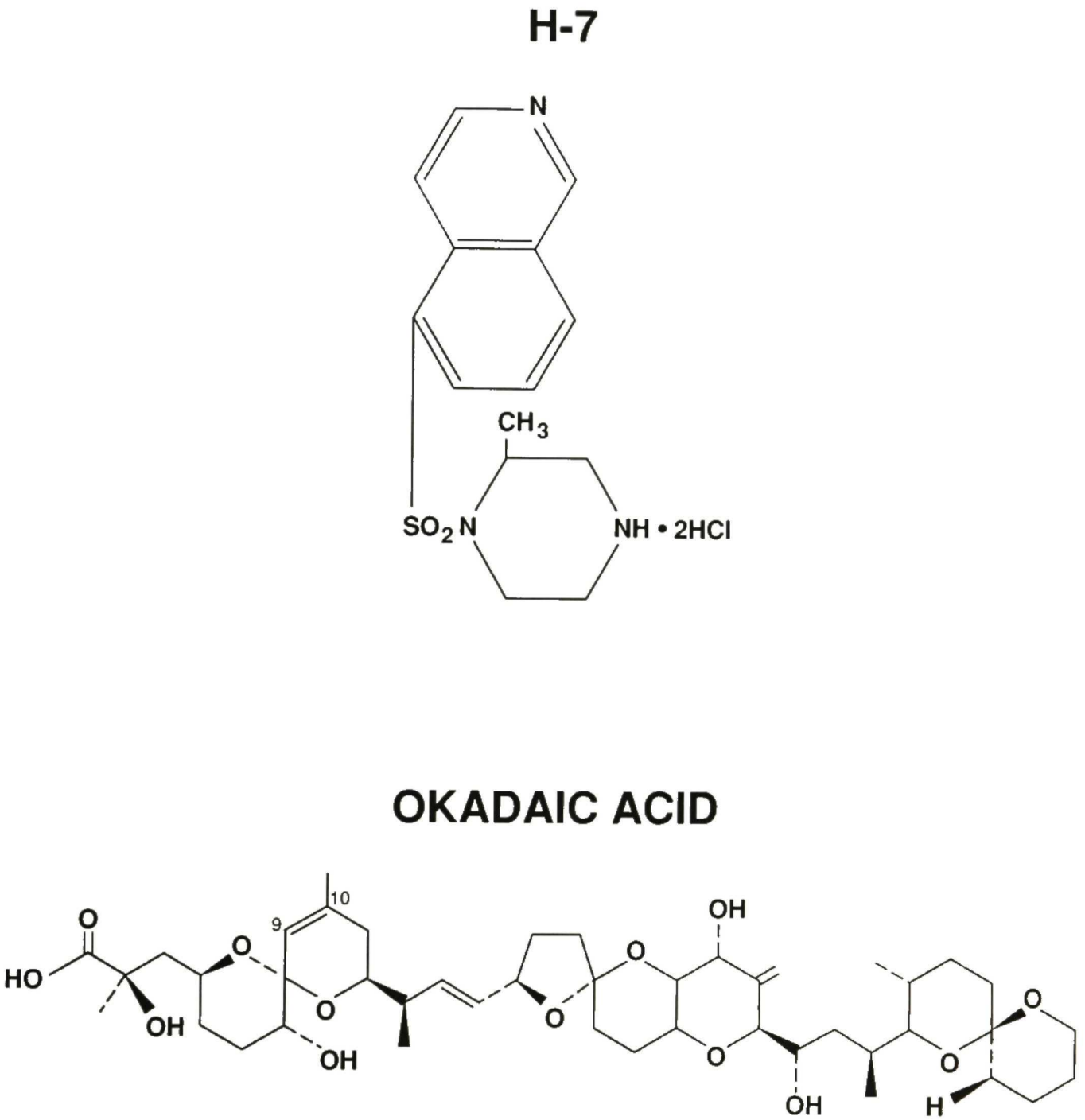

Figure 6.

Chemical structure of H-7: A Kinase Inhibitor

and, Okadaic Acid: A Phosphatase Inhibitor

residues(Haystead et al, 1989).

Okadaic acid is a powerful tumor promoter, and in two-stage carcinogenesis experiments on mouse skin, is of comparable potency to agents like phorbol esters, which exert their effects by activating PKC (Suganuma et al., 1988). Since PP1 and PP2A are likely to be the chief enzymes that reverse the actions of PKC, okadaic acid and phorbol esters probably accelerate tumor formation by increasing the phosphorylation of the same protein(s) (Haystead et al., 1989).

Research Goals:

The aim of this work was to utilize a cellular system in which the 2-5A-depRNase is regulated by IFN and by growth arrest. To study the interaction of PKC with the 2-5A system, the JLS-V9R cell line was chosen, since it prolifererates rapidly and the levels of 2-5A depRNase are induced (10-20) fold with IFN treatment (Jacobsen et al., 1983a). JLS-V9R was derived by chronically infecting an established murine bone marrow cell line (JLS-V9) with Rausher leukemia virus (Allen et al., 1976), and then cloned to obtain a uniform population of cells. The levels of 2-5A depRNase in JLS-V9R cells are highly sensitive to IFNs,

which are growth inhibitors, and PKC activators, which are often growth stimulators. A significant enhancement in the levels of 2-5A-depRNase is also achieved during confluency in this cell line (Jacobsen et al., 1983b). The regulation of 2-5A-depRNase must first be understood before its function in IFN treated and growth arrested cells can be addressed.

Specifically, studies will address:

1. 2-5A-depRNase levels in IFN treated and PKC activated cells.

2. Levels of 2-5A-depRNase in PKC inactivated cells; either by blocking PKC activity with an inhibitor or by chronic exposure to phorbol esters.

3. Determining the level at which regulation of 2-5A-depRNase occurs.

4. Is the 2-5A-depRNase a substrate for PKC ?

5. Are other kinases involved in its regulation ?

6. How does phosphorylation of the 2-5A-depRNase affect its ability to bind 2-5A ?

7. Is 2-5A-depRNase subject to multiple levels of regulation?

The data obtained indicates that 2-5A-depRNase is a physiological substrate for PKC which is phosphorylated in response to mitogens, serum and other growth factors. Phosphorylation of 2-5A-depRNase by PKC, reduces its ability to bind 2-5A, resulting in its transient inactivation, which may allow for the expression of certain proto-oncogenes. 2-5A-depRNase may therefore play a key role as a negative regulatory element in cellular growth control.

MATERIALS AND METHODS

Materials:

Retinoic acid, TPA, H-7, diolein, cholera toxin, forskolin and phosphatidylserine were obtained from Sigma Chemical Co.(St. Louis, MO). Okadaic acid was obtained from Moana Bioproducts, Inc (Honolulu, Hawaii). Murine IFN $\alpha + \beta$ was purchased from Lee Biomolecular (San Diego, CA) and purified PKC was from Lipidex, Inc (Westfield, NJ). PKA and PKA inhibitor were both obtained from Sigma Co. Indolactam was kindly provided by Dr J. Mond (U.S.U.H.S). Cholera toxin was dissolved in H_2O (1 mg/ml); forskolin in methanol (10 mg/ml).

Methods:

Cell Culture.

Murine JLS-V9R cells were cultured in EMEM supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2, 95% air in 150mm flasks. Cells were seeded at $5x10^5$ cells/flask and at sub-confluency were

treated with 1000 u/ml of murine IFN (alpha + beta) for 16 hrs. The stock of TPA was dissolved in ethanol such that the final concentration of ethanol in the culture medium was 0.05%, a concentration at which the solvent had no effect on the morphology of cells or on 2-5A depRNase levels (data not shown). Indolactam and DAG were dissolved in DMSO with a final concentration of DMSO in the culture medium of 0.05%. H-7 was dissolved in H_2O. Diolein and phosphatidylserine were dissolved in chloroform. PKC activators or okadaic acid were added after 16 hrs of IFN treatment while the inhibitor H-7 was added prior to IFN treatment.

In experiments with okadaic acid; cells were seeded as above and treated with IFN for 16 hrs to induce the 2-5A-depRNase; okadaic acid at a concentration of 100 nM was added after the IFN treatment.

Differentiation of Embryonal Carcinoma (EC) Cells

EC cells grown in F-10 media were supplemented with 10% FBS, 1% glutamine, 1% essential and non-essential aminoacids, 1% essential vitamins and 1% pen-strep. Retinoic acid (50 nM) dissolved in ethanol was added to the media at the time of seeding. Media was changed every 48 hrs and cells were allowed to differentiate for 5 to 8 days.

Preparation of cell extracts.

The cells were harvested by scraping and were then washed three times in ice-cold phosphate-buffered saline, and stored as pellets at -70^{0}C. Cells were lysed by thawing into 0.5% Nonidet P-40; 90 mM potassium chloride; 1.0 mM magnesium acetate; 10 mM Hepes, pH 7.6, and 100 ug/ml leupeptin (added fresh). Protein concentrations were determined on whole cell extracts using the Bio-Rad protein assay.

Determination of protein concentration in cell extracts.

"Bio-Rad protein mixture" was prepared by mixing 1 part bio-rad assay dye with four parts of distilled water, and then filtered through Whatman type paper. Standards were prepared by mixing 2, 5, 10, 20, and 30 ul of a 1 mg/ml solution of bovine serum albumin (BSA) with 2.0 ml of diluted dye. Cell extracts were diluted 1:10 by mixing 5 ul of cell extract to 45 ul of distilled water and 5 ul of the diluted cell extract was added to 2.0 ml of the diluted dye. Protein concentrations using the standard curve were determined at A_{595}, in a Beckman spectrophotometer.

Synthesis and purification of $p(A2'p)_2(br^8A2'p)_2A3'[^{32}p]Cp$, (Br-2-5A[^{32}p]Cp)

The bromine-substituted 2-5A derivative, $p(A2'p)_2(br^8A2'p)_2A$ was a kind gift from (P.F. Torrence, Bethesda).

$p(A2'p)_2(br^8A2'p)_2A$ was covalently labeled at its 3'-OH by linking [5'-^{32}p]pCp (3 X 10^6Ci/mole, NEN) with T4 RNA ligase. The reaction catalysed by T4 RNA ligase is:

$$p(A2'p)_2(br^8A2'p)_2A + [5'-^{32}P]pCp + ATP \xrightleftharpoons{\text{T4 RNA ligase, } Mg^{2+}} p(A2'p)_2(br^8A2'p)_2A3'[^{32}p]Cp + AMP + PPi$$

250uCi of [5'-^{32}P]-pCp (> 2,000-4,000 Ci/mmole from NEN, dissolved in 10mM Tricine, pH 7.6) was dried in vacuo using a Speed Vac (Savant). Dried [5'-^{32}p]-pCp was dissolved in 25ul of ligase buffer [100mM HEPES, pH 7.6; 15mM $MgCl_2$; 6.6 mM dithiothreitol; and 20% v/v dimethyl sulfoxide (Mallincrkrodt)]. The tube containing the pCp solution was kept on ice and the following solutions were added:

------ 5ul of (1.0 mM dTTP; 0.1mM ATP, pH 7.0)

------ 10ul of 0.5 mM Br-2-5A in H_2O

------ 8ul H_2O

------ 2ul of T4 RNA ligase (BRL) (18.0 units)

After the reaction mixture was pipetted up-and-down a few times, it was placed in a lead container with ice and refrigerated (2-4^{o}C) for 18hr. During this time the ice melts leading to a gradual acceleration in the rate of the reaction. The reaction mixture was then heated to 90^{o}C for 5 min and centrifuged at 10,000 X g for 10 min in a microfuge. The supernatant was transferred to a new tube.

HPLC purification of Br-2-5A[^{32}p]Cp from the ligase reaction mixture

The unlabeled acceptor molecule, p(A2'p)$_2$(br^{8}A2'p)$_2$A, is present in the reaction mixture in a large molar excess compared to the product, Br-2-5A[^{32}p]Cp; it is therefore necessary to perform an HPLC separation to purify the latter. An HPLC system from Beckman Instruments which includes a model 421 controller, two model 122 solvent modules (pumps) and a model 160 254 nm spectrophotometer,

with a column effluent connected to a model 201 Gilson fraction collector was used for this purpose. The reaction mixture was separated on a reverse phase C-18 column in a linear gradient from 0 to 20% of 1:1 methonol:H_2O in 50mM ammonium phosphate, pH 7.0 at a flow rate of 1.0 ml/min in 20 min. The column is first standardized to determine the time of elution of different oligomers of 2-5A. Five ul of a millimolar solution was injected into the column and eluted (Brown et al.,1981). Once it was established that the system was optimally separating 2-5A oligomers, the injector port was thoroughly washed and two blank runs were performed to remove any traces of 2-5A which could contaminate and thereby reduce the specific activity of the Br-2-5A[^{32}P]Cp. The fraction collector was programmed to collect fractions every 5 min. To locate the peak of Br-2-5A[^{32}P]Cp, 2 ul of each sample was collected into 5 ml of aqueous scintilation fluid, and the radioactivity determined. This HPLC purified Br-2-5A[^{32}P]Cp was used in the photoaffinity labeling of 2-5A-RNase using a UV lamp.

Assay for 2-5A depRNase.

A photoaffinity labeling method was used to detect 2-5-depRNase (Nolan et al., 1989) (Fig. 7).

Figure 7. A schematic of the photoaffinity labeling method used to detect 2-5A-depRNase.

The bromine-substituted 2-5A derivative $p(A2'p)_2(br^8A2'p)_2A$ was covalently labeled at its 3'-OH by linking [5'-^{32}P]pCp (3 X 10^6 Ci/mole) with T4 RNA ligase. The labeled Br-2-5A was then covalently crosslinked to 2-5A-depRNase in extracts of cells with UV light at 308 nm. Analysis of the 77-80 kDa 2-5A-depRNase was by autoradiography after SDS/ polyacrylamide gel electrophoresis.

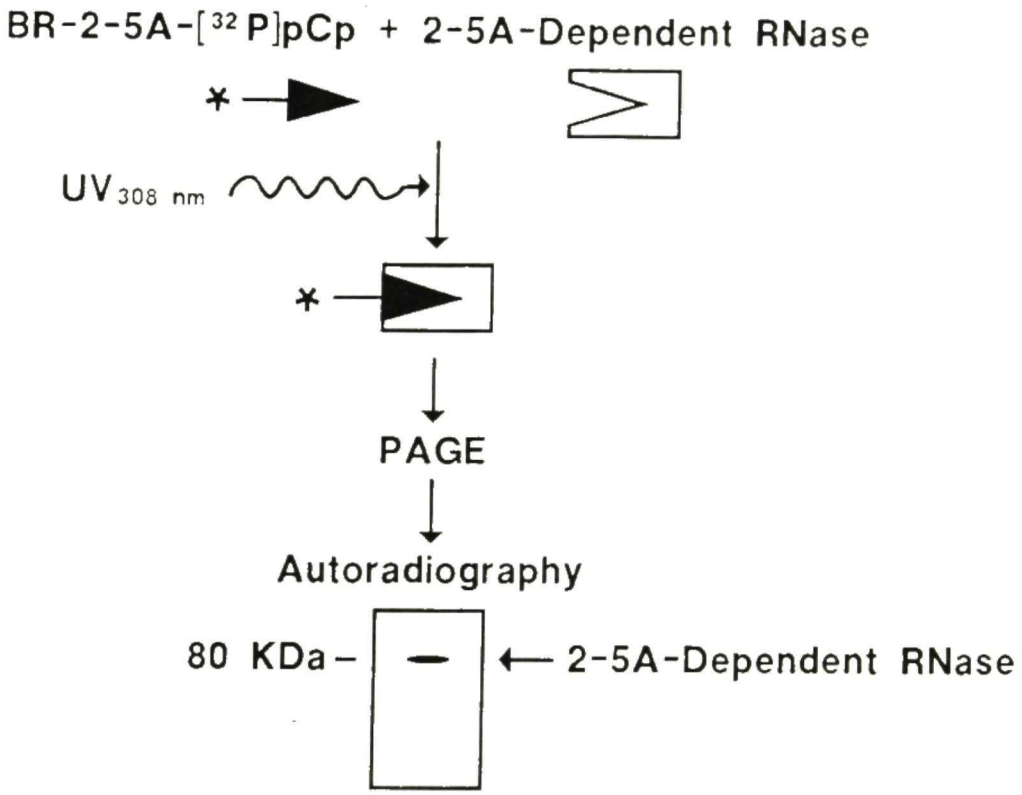

Figure 7.

A schematic of the photoaffinity labeling method used to detect 2-5A-depRNase

The Br-2-5A[^{32}]Cp was covalently crosslinked to 2-5A-depRNase in extracts of cells with UV light at 308nm. Analysis of the labeled, 80 kDa RNase was by autoradiography after SDS/Polyacrylamide gel electrophoresis. Crosslinking was performed in a final volume of 60 ul in CB buffer [100mM HEPES, pH 7.5; 50mM magnesium acetate; 70 mM beta-mercaptoethanol; and 90 mM potassium chloride), containing cell extract (100 ug of protein) and 50,000 counts/min of the probe. Reaction mixtures were preincubated on ice for 30 min. Irradiation was performed at a distance of 5 cm from the bulbs in 96 well microtiter plate placed on ice. After irradiation, reaction mixtures were heated at 100^{o}C for 5 min in Laemmli sample buffer (Laemlli., 1970) and samples were analysed on 10% SDS-PAGE.

<u>Purification of 2-5A-depRNase from mouse liver.</u>

Purification of 2-5A-depRNase was by using a modification of the method of Silverman et al., 1988; (A. Zhou and R.H. Silverman, unpublished). Frozen mouse livers (Swiss-Webster mouse strain purchased from Rockland Inc) were homogenized for 1 min, using a motorized tissue-homogenizer, in 100 ml of homogenization buffer (100 mM HEPES, pH 7.5; 50 mM magnesium acetate; 70 mM beta-mercaptoethanol; 90 mM potassium chloride; 10 mM ATP; 1:50

10 mg/ml leupeptin + 0.5 % NP-40), supplemented with pepstatin (25 ug/ml), trypsin inhibitor III-O (50 ug/ml), trypsin inhibitor I-S (30 ug/ml), leupeptin (100 ug/ml) (Fraction A). The crude lysate (Fraction A), was centrifuged at 8000 rpm for 20 min at 2-4^{o}C, and the supernatant (Fraction B) was removed. Fraction B was centrifuged at 24,000 for 3hr at 2^{o}C and the supernatant (Fraction C) was removed. It is very important that the supernatant be kept on ice. Core(2-5A) was synthesized and covalently attached to hydrazide-sepharose. The core(2-5A)-sepharose suspension was centrifuged at 1,500 rpm for 10 min at 2^{o}C and the supernatant was discarded. The core(2-5A) sepharose, about 1.0 ml of packed volume containing 32 umoles core(2-5A) in AMP equivalents, was washed three-times in 10-volumes of homogenization buffer, supplemented with 0.2 mg/ml leupeptin, a proteinase inhibitor. The washed core(2-5A)-sepharose was added to fraction C, and the suspension was mixed by magnetic stirring overnight at 2^{o}C. The Sepharose was then pelleted by centrifuging at 3500 rpm for 10 min, and was then washed three times in 100 ml of homogenization buffer, supplemented with 0.2 mg/ml leupeptin. A column was made by using poly-prep column (Bio-Rad), and washing was continued with 0.5 X CB + 1 M KCl. The column was washed successively in the following order.

_____ 0.5 X CB + 2 M KCL for 10 min.

_____ Buffer A [Tris-HCl, 20 mM; KCl, 50 mM; $Mg(OAc)_2$,

5 mM; EDTA, 1 mM; beta-mercaptoethanol, 8 mM; Glycerol 5%].

_____ 0.3 M Guandine-HCl for 10 min.

_____ Elute 2-5A-depRNase with 3 M Guandine-HCl.

Collected fractions were dialysed overnight against buffer A. A photochemical crosslinking assay was performed to determine the optimal 2-5A-depRNase fraction.

Linkage of tetramer core to hydrazide-sepharose

Linkage of tetramer core to hydrazide "adipic acid" sepharose was by a modification of the method of Silverman and Krause, 1986 (A. Zhou and Silverman. R, unpublished). 1.7 ml of 1.0 mM tetramer core was oxidized by adding 130 ul of 146 mM sodium metaperiodate (Sigma Co.). Excess periodate was quenched by adding 1/9th volume (200 ul) of 0.6 M ethylene glycol/50 % glycerol. 6 ml of hydrazide-sepharose (Pharmacia Inc.) was washed three times and resuspended in 0.6 ml of 0.1 M sodium acetate (pH 5.5). The oxidized 2-5A solution was added to the sepharose suspension, mixed briefly and incubated at 25^{o}C for 30 min. The mixture was then agitated with magnetic stirring at 2-4^{o}C for 36 hrs.

The core-sepharose pellet was washed 5 times in 2 X SSC after centrifuging and resuspended in 1 volume of 2 X SSC and 2 volumes ethanol and stored at -20^{o}C. Concentration of the core bound to the sepharose was determined spectrophotometrically after alkaline hydrolysis (Silverman and Krause, 1986).

<u>In vitro phosphorylation of 2-5A-depRNase by PKC and PKA.</u>

PKC phosphorylation of partially purified 2-5A-depRNase in 80 ul final volume of reaction mixture containing 2 ng of purified PKC (2.8 mol/min/mg protein of phosphate transfering activity containing < 1% lipid -independent kinase activity), 20mM Tris (pH 7.5), 5mM MgOAc, 100 M $CaCl_2$, 0.25 ug phosphatidyl serine, 0.025 ug Diolein, 10 uCi [gamma-^{32}P]ATP and 1 uM unlabeled ATP. Reaction was heated to 30^{o}C for 5 min, and was stopped by adding Laemmli sample buffer. Samples were analysed on 10% SDS-PAGE.

PKA phosphorylation of partially purified 2-5A-depRNase in 100 ul final voulume of reaction mixture containing, 50 uM sodium acetate; 10 uM MgOAc; 5 nM cAMP; 2 uM (20 uCi) {gamma-^{32}P}ATP. Histone 40 ug was included as a positive control. Reactions were heated to 30^{o}C for 5 min and analysed as above.

Extraction of 2-5A from intact cells:

This technique is a modification of previously described techniques and is designed for monolayer cultures of cells (Silverman et al., 1981; Hersh et al., 1984; Silverman and Krause., 1986). Culture medium was removed from monolayers of cells, and the cells were washed twice in ice-cold phosphate buffered saline. Two ml of 10% TCA was added directly to the plates and the cells were scraped using a rubber policeman. Cell extract was collected in a 4.0 ml centrifuge tube, vortexed vigorously and placed in an ice bath for 10 min. After vortexing a second time, the extract was centrifuged at 750 X g for 5 min at 2-4^{o}C. The supernatant was transfered to a polypropylene centrifuge tube, and the volume adjusted to 2.0 ml with 10% TCA. The pellet was saved for a protein determination. Freon (1,1,2,-trichloro-1,2,2,-trifluoro-ethane, Mallinckrodt) and tri-n-octylamine (Sigma Co.) mixed in a ratio of 39:11 was added to the supernatant. This mixture was vortexed for 15 sec and then centrifuged at 3,500 X g for 5 min at 2-4^{o}C. The upper aqueous phase was transfered to a clean polypropylene tube, and an equal volume of the Freon:tri-n-octylamine mix was added, vortexed and centrifuged as above. The upper aqueous phase was collected and the pH was adjusted to between 6.5 - 7.5 with HEPES, pH 7.0.

Radiobinding assay for detecting 2-5A.

A "Br-2-5A[^{32}P]Cp containing mixture" was prepared by mixing 5,000 cpm of HPLC purified Br-2-5A[^{32}P]Cp in 10mM Tris-HCl pH 7.6, 2mM magnesium acetate, 0.4 mM ATP pH 7.0, and 2% v/v glycerol in a ratio of 1:10. Incubations were set up in 0.5 ml conical tubes using the following in each reaction mix.

------ 10 ul Br-2-5A[^{32}P]Cp

------ 5 ul competitor (Sample from cell extract)

----- 5 ul (100 ug) of postmitochondrial mouse L cell extract to each tube (the source of 2-5A-depRNase).

A standard curve using authentic 2-5A was performed with each assay using 5 ul of 1.25, 2.5, 5, 10, 20, 40, 80, and 250 nM of trimer 2-5A. The assay tubes were incubated on ice over night at 2-4^{O}C. 2.5 cm diameter nitrocellulose discs (Millipore Co., HAWP 02500, 0.45 um pores) were cut in half and numbered with a water insoluble ink. Each assay mixture was spotted on the filters, placed on a sheet of aluminum foil. It is critical that this step be done quickly to avoid drying of the filters. Immediately after spotting, the filters were placed in a beaker containing 10 ml tap

distilled H_2O per filter. The filters were gently swirled for 30 seconds and the water discarded. A second water rinse for 30 seconds followed. The filters were removed, spread on a new piece of aluminum foil, and dried using a heat lamp for 15 min. The filters were placed in counting vials to which was added 5 ml of scintilation fluid and radioactivity was determined (Knight et al., 1980).

In vitro transcription of the cDNA coding for 2-5A-RNase and translation in wheat germ extract.

A cDNA clone coding for 2-5A-depRNase was isolated (B. Hassel, A. Zhou, and R.H. Silverman, unpublished), in vitro transcribed and the corresponding mRNA was in vitro translated in cell free extracts of wheat germ.

In vitro transcription.

Incubations were set up in 0.5 ml conical tubes using the following in each reaction mix:

cDNA coding for 2-5A-depRNase (7 ul) was mixed with:

------- 10 ul 5 x sample buffer (Promega)

------- 12 ul (3 ul x 4 dNTP 10 mM)

------- 2.0 ul 0.75 mM DTT

------- 2.0 ul RNasin (40 u/ml)

------- 2.0 ul T_3 RNA Polymerase (26 u/ml)

------- 10.0 ul Diethyl-pyrocarbonate treated H_2O

This mixture was incubated at 37^{o}C for 1 hr, then treated with DNase I for 30 min at 37^{o}C to digest template. RNA was extracted with one volume of phenol/chloroform and precipitated at -20^{o}C with 2.5 volumes of EtOH.

<u>In vitro translation.</u>

In vitro transcribed RNA was translated in wheat germ extract as follows:

------- 25 ul wheat germ extract

------- 10 ul H_2O

------- 1 ul RNasin ribonuclease inhibitor (40 u/ml)

------- 7 ul 1mM amino acid mixture

------- 1 ul 1M potassium acetate

------- 2 ul RNA substrate in H_2O

---

50 ul [Final volume]

Template mRNA was heated at 67^{o} for 10 min and immediately cooled on ice. This increases the efficiency of translation, especially of GC-rich mRNA, by destroying local regions of secondary structure. Residual ethanol was removed from mRNA preparations and labeled amino acids were added to the translation reaction. The reaction components were assembled and the lysate was gently vortexed upon addition of each component The translation reaction was incubated at 25^{o} for 60 min. Translation product was analysed using Br-2-5A[^{32}p]Cp in a covalent binding assay.

RESULTS

Induction of 2-5A-depRNase during differentiation of embryonal carcinoma cells with retinoic acid (Figure 8).

The highly sensitive photoaffinity method was used to study regulation of 2-5A-depRNase in EC and JLS-V9R cells. Previous studies have demonstrated that treatment of EC cells with retinoic acid, dbcAMP and IBMX differentiates them from small indistinct cells to flat endodermal like cells (Krause et al., 1985). This morhological change is accompanied by growth inhibition and a subsequent enhancement in the level of the 2-5A-depRNase, implicating a possible role for this enzyme in differentiation. I analyzed the effect of retinoic acid alone on the levels of 2-5A-depRNase and the morphological changes in EC cells.

PC-13, an EC cell line which proliferates rapidly, has very low basal levels of 2-5A-depRNase. Cells grown in F-10 media supplemented with 10 % fetal bovine serum were treated with 50 nM retinoic acid for 5 days at 37^{o}C. Cells were lysed in NP-40 lysis buffer and 2-5A-depRNase levels were measured by using the highly sensitive photoaffinity method (Fig. 8). Unlabeled 2-5A was used in lanes 2, 4, and 6 to show the specificity of the probe for binding to the RNase.

Very low basal levels of 2-5A-depRNase was observed in undifferentiated PC-13 cells(lane 1); IFN failed to induce 2-5A-depRNase(lane 3); with 5 days of retinoic acid treatment a 20 fold induction in the RNase was observed. An induction in the RNase was followed by growth arrest within 7 to 8 days of retinoic acid treatment.

Figure 8. Induction of 2-5A-depRNase during differentiation of embryonal carcinoma cells with retinoic acid.

2-5A-depRNase assays were performed as described (Materials amd Methods) on PC-13 cell lysates from untreated cells (lanes 1 and 2); cells treated with 1000 u/ml murine α + β IFN for 7 days (lanes 3 and 4); or cells treated with retinoic acid, 50 nM for 7 days (lanes 5 and 6). Unlabeled 2-5A was used as a competitor in lanes 2, 4, and 6 to show the specificity of the probe for binding to the 2-5A-depRNase.

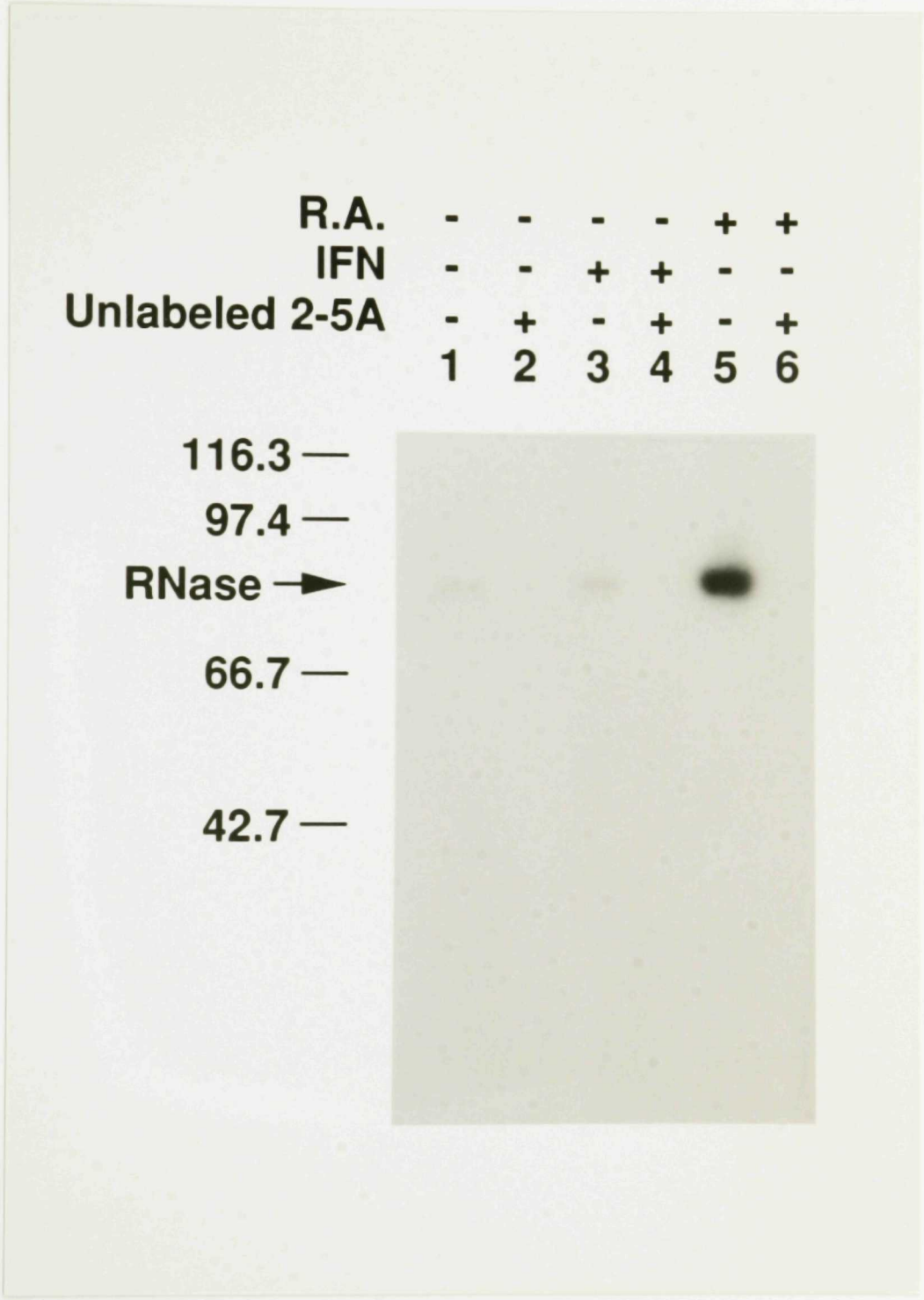

Figure 8.

Induction of 2-5A-depRNase during differentiation of embryonal carcinoma cells with retinoic acid.

The effect of Retinoic acid on PC-13 morphology (Figure 9). [Magnification: x 20]

A. Untreated 5 day control cultures.

EC cells were grown in monolayers as described in materials and methods. These cells are multipotential stem cells that grow in tight clusters as "epitheliod nests", and proliferate rapidly (Martin, G., 1980).

B. After 5 days of treatment with retinoic acid.

PC-13 cells were treated with 50 nM retinoic acid and photographed 5 days post treatment. The media containing 50 nM retinoic acid was changed every 48 hr. Treating PC-13 cells with 50 nM retinoic acid, a morphological alteration from small, rounded cells in "epithelioid nests" to large, flat cells was observed. Perinuclear and cytoplasmic granules with nerve like processes was also striking. Differentiation was followed by an enhancement in the levels of 2-5A-depRNase, as described previously (Fig 8), and a subsequent arrest in proliferation.

Figure 9. The effect of retinoic acid on PC-13 morhology.

A. Untreated 5 day control cultures.

B. PC-13 cells were treated with 50 nM retinoic acid and photographed 5 days post-treatment. Media was changed every 48 hrs.

A.

B.

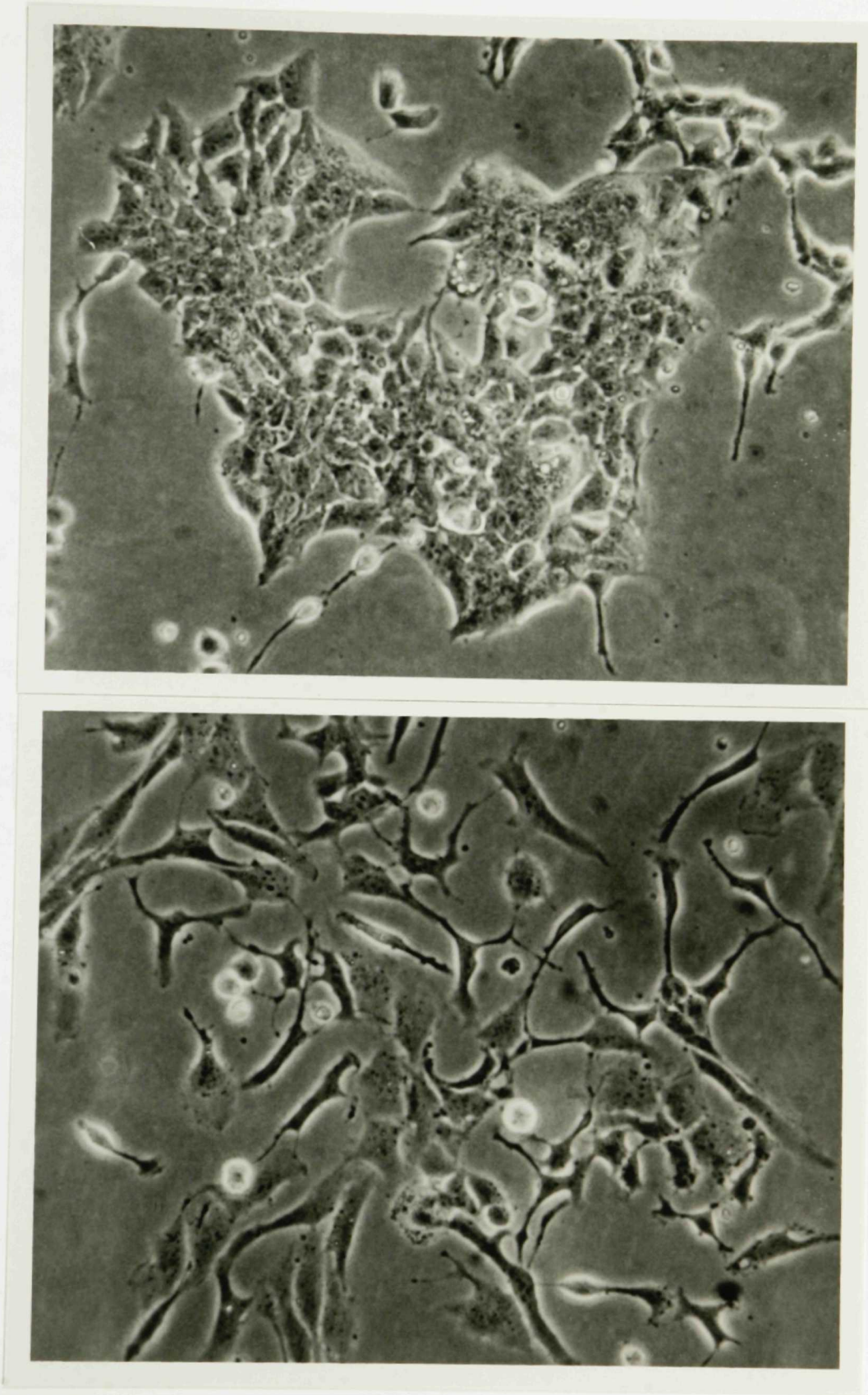

Figure 9.

The effect of Retinoic acid on PC-13 morphology

Activators of PKC, down-regulate 2-5A-depRNase; as determined by its ability to bind Br-2-5A[^{32}P]Cp (Figure 11).

Studies by (Jetten and Luca. 1982; Sporn and Roberts 1983) have shown that, in many cell systems, retinoids are antagonistic toward the effects of PKC activators such as phorbol ester tumor promoters. Since retinoids have been used extensively to differentiate and inhibit growth of cells (Martin, 1980; Krause et al., 1985), it was of interest to establish if PKC activators altered levels of 2-5A-depRNase, which has been shown to be enhanced during growth arrest and differentiation. I therefore investigated the effect of PKC activators and inhibitors on 2-5A-depRNase levels.

JLS-V9R cells, a murine bone marrow derived cell line was chosen for this study as significant fluctuations in the level of 2-5A-depRNase is achieved during growth arrest and interferon treatment. EC cells were not suitable because PKC activators had no effect on morphology or 2-5A-depRNase levels once differentiation and subsequent growth arrest was achieved (data not shown). A possible explanation for this may be that with terminal differentiation, PKC is translocated to the cytosol and is no longer responsive to the growth stimulatory effects of mitogens.

JLS-V9R cells which contain very low basal levels of 2-5A-depRNase were treated at sub-confluency with 1000 u/ml of murine α + β IFN for 16 hrs. This resulted in a 10 fold induction in the levels of 2-5A-depRNase (Fig. 10). PKC activators TPA, Indolactam and DAG were then added for 10, 30, 60, 120, 180, and 240 min and cells were harvested (Fig. 11). A 3 to 4-fold decrease in 2-5A binding was observed from 30 to 120 min. This data suggests that 2-5A-depRNase is a substrate for PKC, and 2-5A may have recognized only the non-phosphorylated form of 2-5A-depRNase. Alternatively, an induction of endogenous 2-5A or analogs of 2-5A could have competed with the Br-2-5A[^{32}p]Cp for binding to 2-5A-depRNase.

Figure 10. Induction of 2-5A-depRNase in murine JLS-V9R cells with IFN treatment. Untreated cells (lane 1). A 10 fold induction was achieved when cells were treated with 1000 u/ml of murine alpha + beta IFN for 16 hrs at subconfluency (lane 2). In subsequent experiments, similar induction in the levels of 2-5A-depRNase was obtained with IFN treatment.

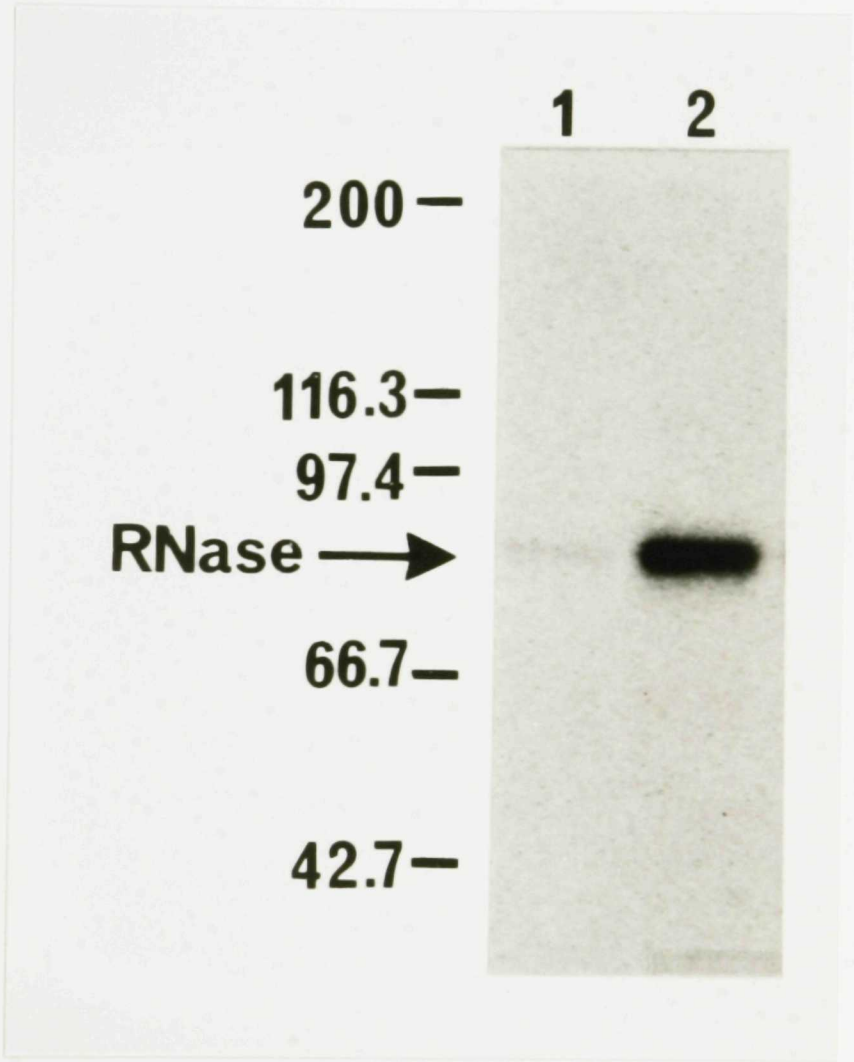

Figure 10

Induction of 2-5A-depRNase in IFN treated JLS-V9R cells

Figure 11. Activators of PKC, down-regulate the 2-5A-depRNase, as determined by its ability to bind Br-2-5A[^{32}p]Cp.

JLS-V9R cells were treated at sub-confluency with 1000 u/ml α + β IFN for 16 hrs to induce the RNase (lane 1). PKC activators TPA (40 nM), Indolactam (1 uM), and DAG (1 uM) were added following induction of the RNase for the times indicated (lanes 2-7).

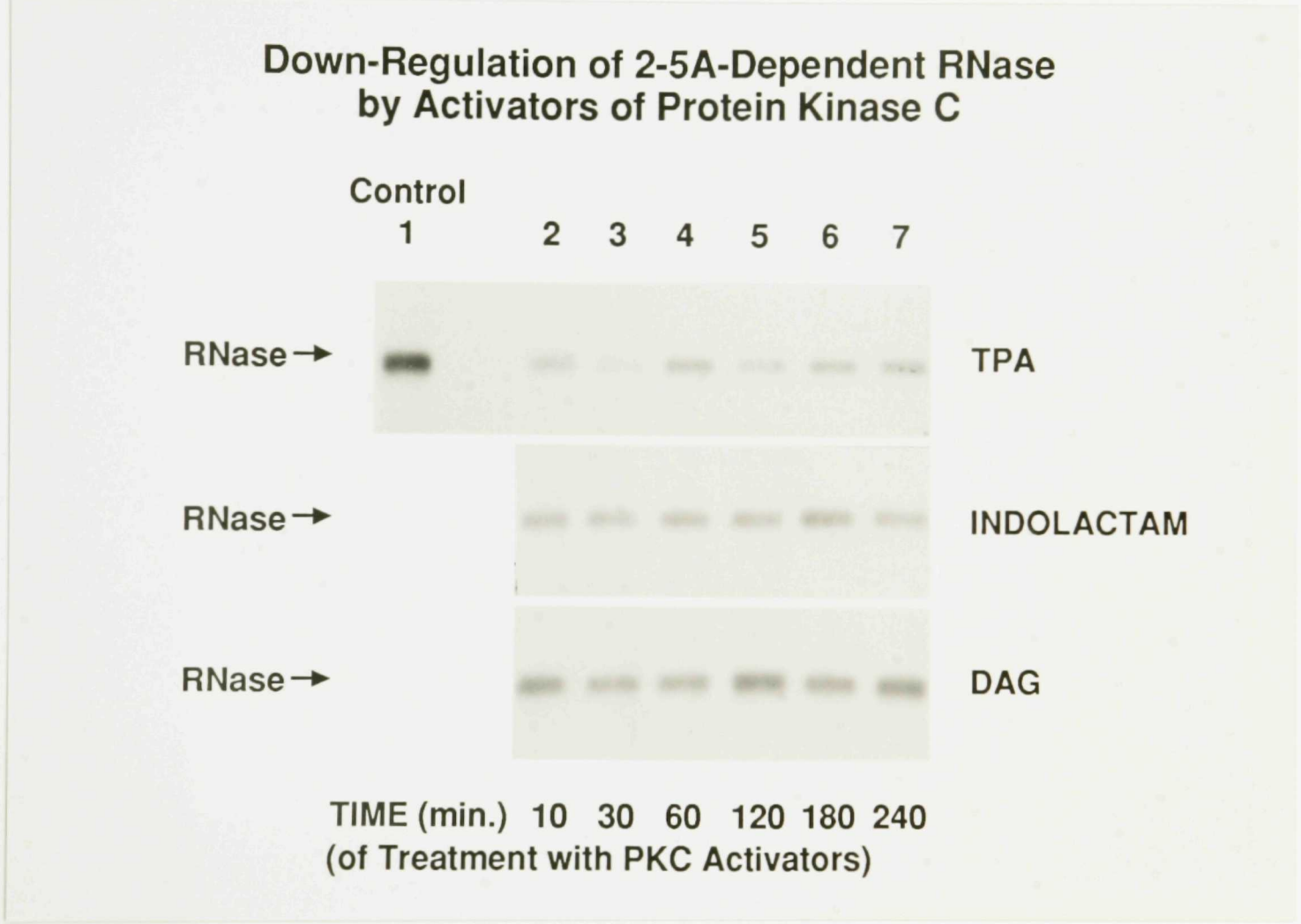

Figure 11.

Activators of PKC, down-regulate the 2-5A-depRNase, as determined by its ability to bind Br-2-5A[^{32}p]Cp

Histogram showing the effect of PKC activators on IFN induced 2-5A-depRNase levels as a function of time (Figure 12).

Quantitation from the autoradiogram shown in figure 11 was done using a betagen counter. Down-regulation of 2-5A-depRNase was most significant at 30 min to 1hr with TPA the most potent agent followed by Indolactam and DAG.

2-5A-depRNase levels in PKC depleted cells (Figure 13)

Since activators of PKC produced a down-regulation in the levels of 2-5A-depRNase, as measured by its ability to bind Br-2-5A[^{32}P]Cp, I investigated the possibility that an enhancement in the 2-5A-depRNase levels could be achieved by depleting PKC with chronic exposure of cells to TPA (Helper et al., 1988). To down-regulate PKC, cells were first treated with TPA (40 nM) followed with IFN-treatment for 16 hrs. Quantitation of the autoradiogram showed a two fold induction in the levels of 2-5A-depRNase after 32 hrs of TPA treatment (lane 2). This result was consistent with the findings of Helper and co-workers (1988) that chronic exposure of cells to phorbol esters results in down-regulation of protein kinase C and a concomitant loss of phorbol ester responsiveness.

Figure 12. Histogram showing the effect of PKC activators on IFN-induced 2-5A-depRNase levels as a function of time.

Quantitation from the autoradiogram shown in figure 11 was done using a betagen counter. A 10 fold induction in the levels of 2-5A-depRNase is first achieved with IFN treatment. With the addition of PKC activators following RNase induction, a 5 fold decrease in 2-5A binding was observed at 30 min with TPA treatment; a 3 and 2.5 fold decrease with IND and DAG at 120 and 60 min. TPA was the most potent downregulator followed by IND and DAG. PKC activators in the absence of IFN had no effect on the basal levels of the RNase (data not shown).

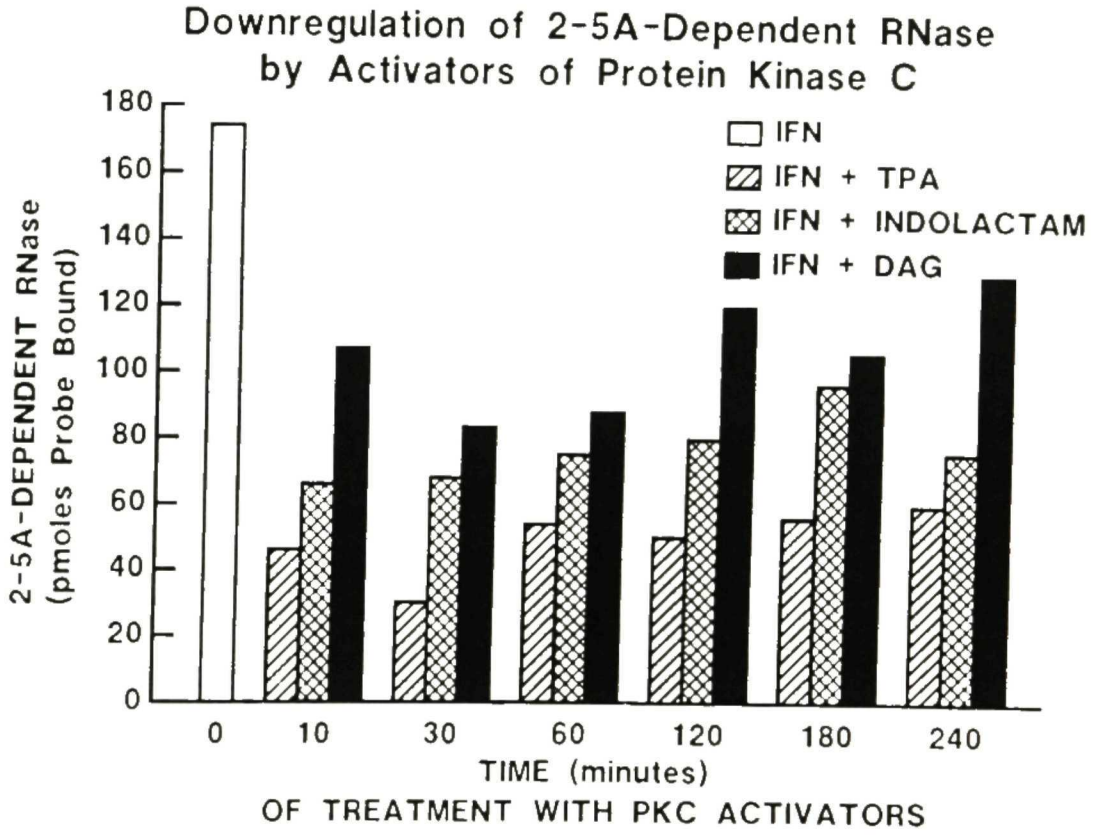

Figure 12.

Histogram showing the effect of PKC activators on IFN-induced 2-5A-depRNase levels as a function of time.

Figure 13. 2-5A-depRNase levels in PKC depleted cells

JLS-V9R cells were treated with IFN for 16 hrs as before to achieve a 10 fold induction in the level of the RNase (lane 1). To down-regulate PKC activity at the membrane, cells were first treated with TPA (40 nM), for 16 hrs, followed by IFN treatment for an additional 16 hrs (lane 2). Betagen quantitation showed a 2 fold induction in 2-5A-depRNase levels.

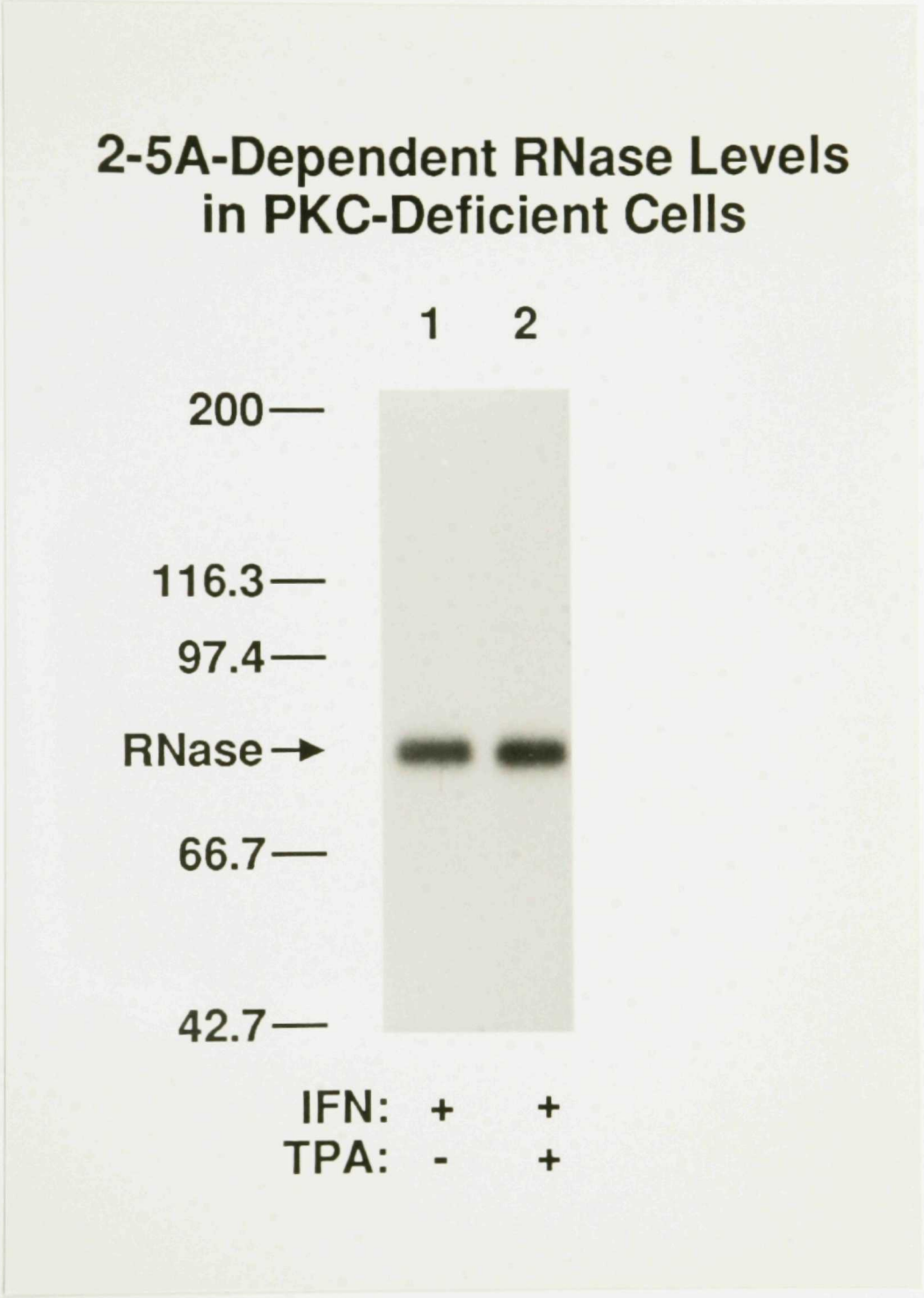

Figure 13.

2-5A-depRNase in PKC depleted cells

Superinduction of 2-5A-depRNase with H-7, a partially selective inhibitor of PKC (Figure 14).

The contribution of PKC to 2-5A-depRNase regulation, can also be examined by the blockade of its activity. Blocking PKC activity, 4hrs prior to the addition of IFN with the isoquinolonesulfonamide derivative, H-7 (30 uM), a partially selective inhibitor of PKC, resulted in a 4.5 fold superinduction of 2-5A-depRNase. However, H-7 (30 uM for 16 hrs) in the absence of IFN failed to induce the RNase in the JLS-V9R cells. Quantitation of the results shown in figure 14 was used to produce a histogram (Figure 15). Bar 1 (Fig. 15) shows the low basal level of 2-5A-depRNase present in the JLS-V9R cells. A 15 fold induction is achieved with 1000 u/ml of murine α + β IFN (bar 2) and a further 4.5 fold superinduction with 4 hr of H-7 treatment (bar 7).

The data obtained here is in support of the hypothesis that activation of PKC with short term treatment with TPA and Indolactam results in the down-regulation of 2-5A-depRNase, whereas, blocking PKC activity caused an enhancement in its level.

Adding H-7, after IFN treatment, however, failed to generate any further induction in 2-5A-depRNase (data not shown). One explanation for this may be that, IFN may itself

have blocked various growth signals through the PKC-dependent pathway. Second, the concentration of IFN (1000 u/ml) used in these experiments may have blocked the permeability of H-7 by occupying receptors that may have caused a membrane alteration.

One interpretation of the data presented thus far is that 2-5A-depRNase is a substrate for PKC. However, less than a two fold superinduction in the 2-5A-depRNase level was observed when PKC was down-regulated with chronic exposure of cells to phorbol esters. In contrast, pre-treatment with H-7 caused a larger 4 fold superinduction in the levels of 2-5A-depRNase. Since H-7 may non-selectively inhibit other kinases, there may be other kinases involved in the down-regulation of the 2-5A-depRNase. Also, another possibility that should be entertained is that, in the JLS-V9R cells a 32 hr exposure to TPA may be inadequate to fully down-regulate the RNase. A possible involvement of other kinases is supported by earlier work of Bloom and co-workers (1982), showing that in transformed macrophages, stimulation of phagocytosis and inhibition of growth by IFN are mediated through intracellular cAMP. Fuse and Kuwata (1978) found a temporal relationship between the increase in intracellular cAMP and growth inhibition in IFN-sensitive cells. Of interest was their finding that IFN-resistant cells, which showed no increase in intracellular cAMP, were not subject

to growth inhibition by IFN, suggesting a correlation between growth inhibition and intracellular cAMP levels. The growth of IFN-resistant cells was inhibited by the addition of exogenous cAMP. In JLS-V9R cells, exposure to IFN and forskolin or cholera toxin, which are agents that increase the intracellular cAMP levels resulted in a superinduction of the 2-5A-depRNase (Figure 16). Since cAMP elevating agents such as dbcAMP, forskolin and cholera toxin have been shown to inhibit growth in several cell lines, and that dbcAMP may act synergistically with retinoic acid in enhancing levels of 2-5A-depRNase in EC cells, it may be that the regulation of 2-5A-depRNase is dictated by both PKC and PKA. Furthermore, since the level of 2-5A-depRNase fluctuates with growth, a role for cdc2 kinase, which regulates cell cycle (Riabowol et al., 1989) is also a possibility.

Figure 14. Superinduction of 2-5A-depRNase with H-7

Murine JLS-V9R cells were pre-treated with 30 uM H-7, a kinase inhibitor, for 1, 2, 3, or 4 hrs prior to the addition 1000 u/ml α + β IFN for 16 hrs.

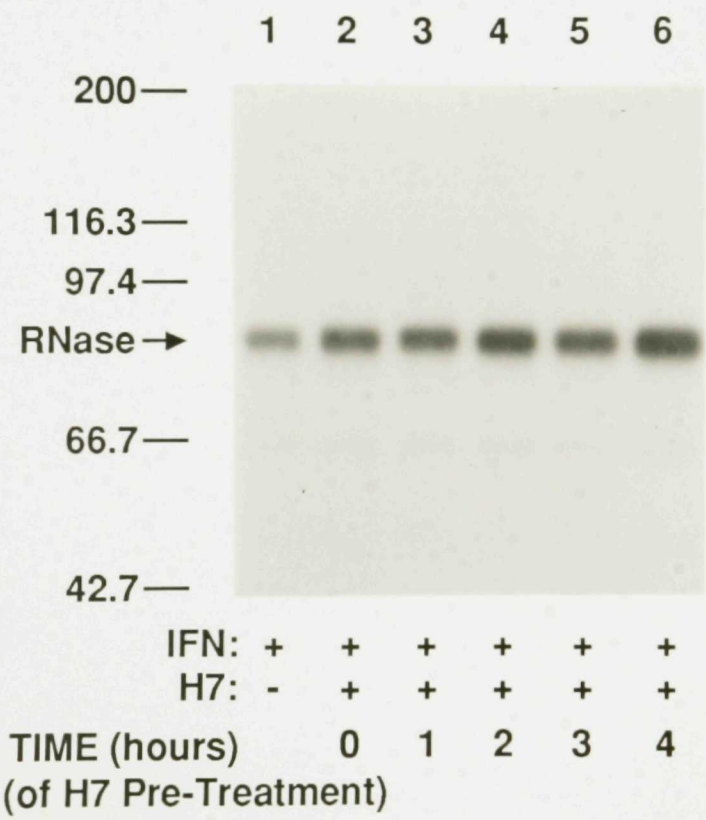

Figure 14.

Superinduction of 2-5A-depRNase with H-7. a partially selective inhibitor of PKC.

Figure 15. Histogram showing the time course effect of H-7 pre-treatment on the levels of 2-5A-depRNase.

Quantitation of the data from figure 14 showed a 4 fold superinduction in 2-5A-depRNase levels when cells were pre-treated with H-7 for 4 h prior to the addition of IFN. H-7 in the absence of IFN however, had no effect on the basal levels of the RNase (data not shown).

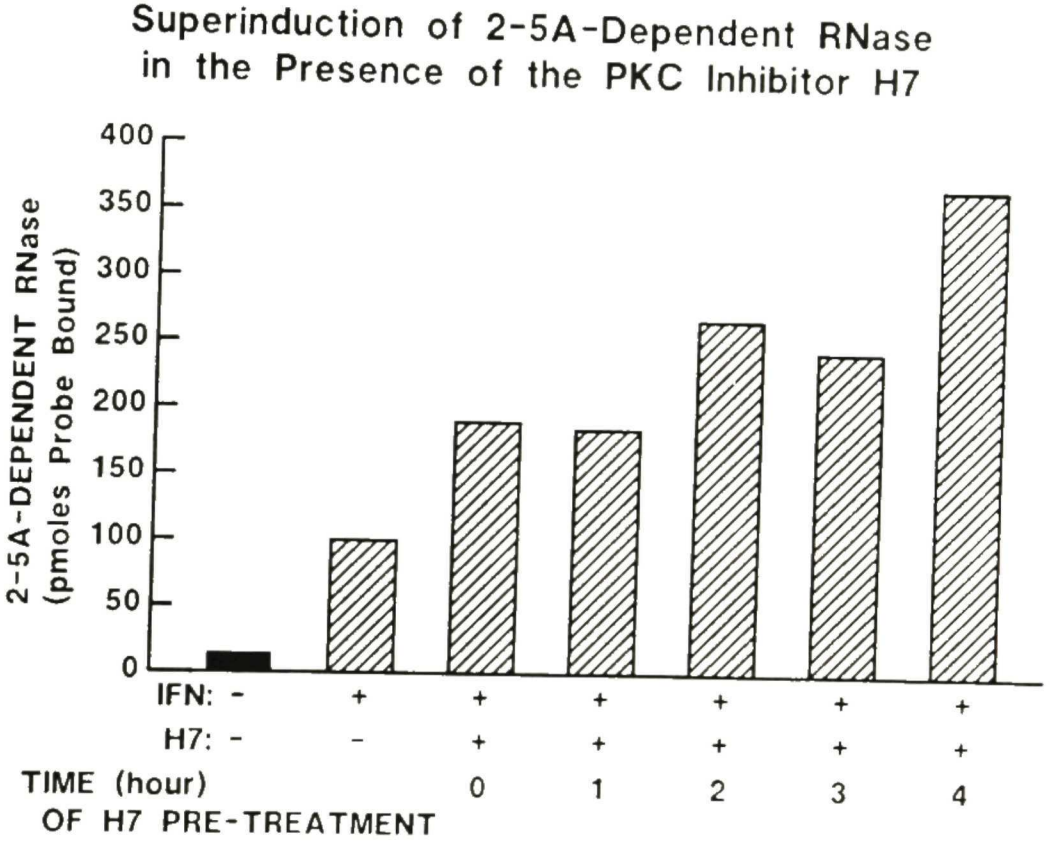

Figure 15.

Histogram showing the time course effect of H-7 pre-treatment on the levels of 2-5A-depRNase

Figure 16. Superinduction of 2-5A-depRNase in IFN-treated, JLS-V9R cells, after exposure to cAMP elevating agents: Forskolin and Cholera toxin.

JLS-V9R cells were pre-treated with forskolin (20 uM) and cholera toxin (20 nM) for 1 h prior to the addition of IFN. IFN treatment was for 16 h as described in experimental procedures. Densitometric analysis showed a superinduction of 3 fold with cholera toxin and 5 fold with forskolin (lanes 6 & 10). Cholera toxin and forskolin in the absence of IFN failed to induce the RNase (lanes 4 & 8). Unlabeled 2-5A was used as a competitor to show the specificity of the probe for binding to the 2-5A-depRNase (lanes 1, 3, 5, 7 & 9). Basal level of RNase was as shown in figure 10, with a 10 fold induction after IFN treatment (lane 2).

Figure 16.

Superinduction of 2-5A-depRNase in IFN-treated, JLS-V9R cells, after exposure to cAMP elevating agents: Forskolin and Cholera toxin

Effects of Okadaic acid treatment on 2-5A-depRNase levels *in vivo.* (figure 17)

To support the theory that 2-5A-depRNase may exist as a phosphoprotein and that it may be subject to a post-translational regulation *in vivo*, studies were performed using okadaic acid, a phosphatase inhibitor. Treating cells with okadaic acid, resulted in a significant down-regulation of 2-5A-depRNase. RNase was induced as before by treating cells at sub-confluency with 1000 u/ml of IFN for 16 hrs, followed by okadaic acid (100 nM) treatment. As with PKC activators, a 3.5 fold down-regulation in the levels of 2-5A-depRNase was observed after 4 hrs (lane 5).

Okadaic acid produces marked increases in the phosphorylaton states of many proteins in adipocytes and hepatocytes, as judged by increased ^{32}P-labelling, without changing the specific radioactivity of intracellular ATP or the ATP:ADP ratio (Haystead et al., 1989). It rapidly stimulates protein phosphorylation in intact cells, and behaves like a specific protein phosphatase inhibitor, with phosphatases PP1 and PP2A as its major targets, which are the chief phosphatases that reverse the actions of PKC (Cohen, 1989).

Figure 17. Down-regulation of 2-5A-depRNase by Okadaic Acid: A phosphatase inhibitor

2-5A-depRNase was induced in the JLS-V9R cells as described in material and methods, followed by okadaic acid (100 nM) treatment for 1, 2, 3 & 4 hrs.

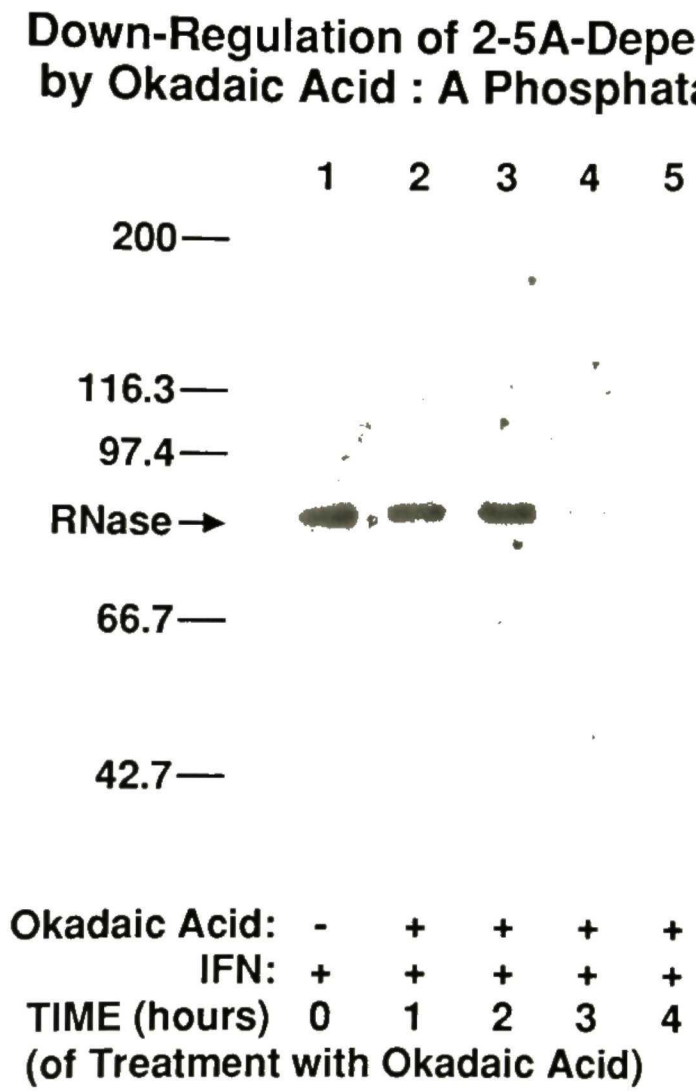

Figure 17.

Down-regulation of 2-5A-depRNase by Okadaic Acid: A phosphatase inhibitor.

The effects of okadaic acid on JLS-V9R cells strongly supports the view that, 2-5A-depRNase is a phosphoprotein, and its phosphorylation status may determine its ability to bind 2-5A. This data further emphasizes a role for 2-5A-depRNase as a growth regulator, as growth stimulation and tumor promotion presumably stem from increased phosphorylation of one or more proteins that are substrates for PKC and dephosphorylated by PPI/PP2A.

Histogram showing the effect of okadaic acid in JLS-V9R cells (figure 18).

Quantitation of 2-5A-depRNase levels from figure 17, using a betagen scanner, showed a 2 fold down-regulation at 3 hrs and 3.5 fold at 4 hrs of post treatment with okadaic acid.

Figure 18. Histogram showing the effect of okadaic acid in JLS-V9R cells.

Quantitation of 2-5A-depRNase levels from figure 17, using a betagen scanner, showed a 2 fold down-regulation at 3 hrs (lane 4), and 3.5 fold at 4 hrs (lane 5). Cell viability (as determined by counting a 1:100 dilution of the cell suspension using a hemacytometer) was unaffected and okadaic acid in the absence of IFN had no effect on the basal level of 2-5A-depRNase (data not shown).

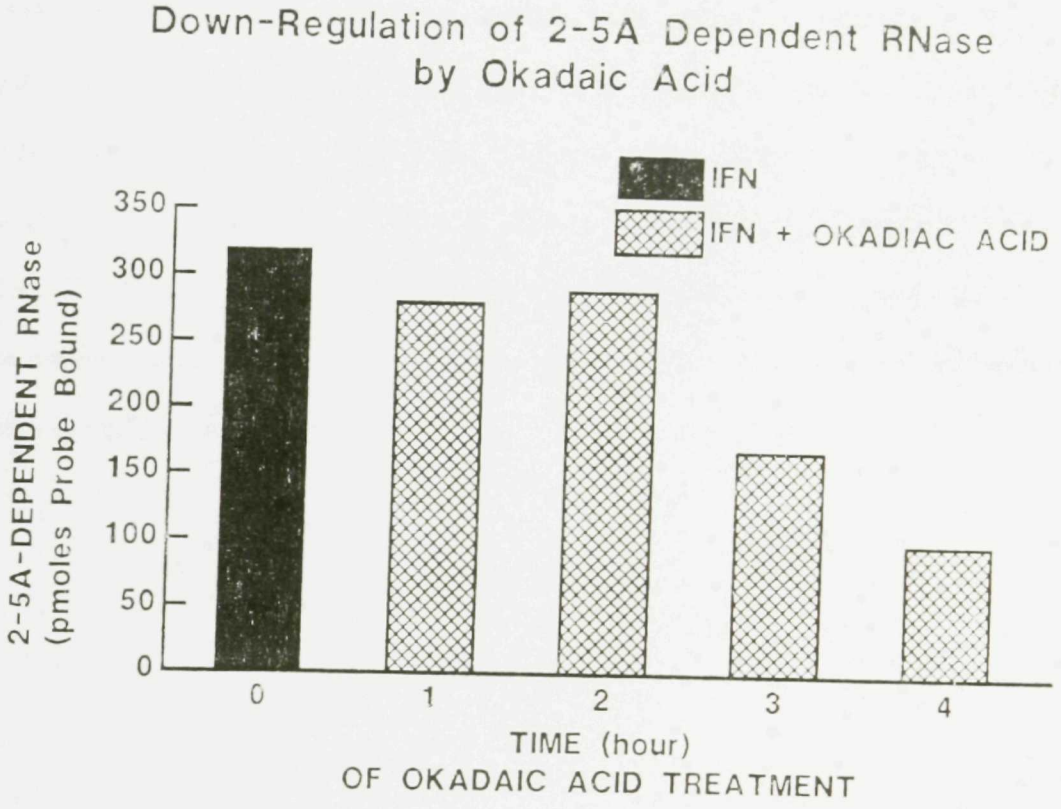

Figure 18.

Histogram showing the down-regulation of 2-5A-depRNase by Okadaic acid: A phosphatase inhibitor

"Mixing Experiment" to determine if any inhibitory factors were induced in vivo with TPA or DiC8 treatment, (figure 19).

Extracts from IFN-treated and TPA-treated cells were mixed to determine if endogenous 2-5A, analogs of 2-5A or other inhibitory factors were induced to cause a down-regulation in 2-5A-depRNase. The levels of 2-5A-depRNase obtained were additive implying that TPA did not induce any significant material which could compete with or inhibit Br-2-5A[^{32}P]Cp from binding to 2-5A-depRNase. Induction of a protease was also unlikely, since no breakdown products of 2-5A-depRNase were seen.

Figure 19. "Mixing Experiment" to determine if any inhibitory factors were induced <u>in vivo</u> with TPA and DiC8 treatment.

Extracts form IFN-treated and TPA or DiC8 (an analog of DAG) treated JLS-V9R cells were mixed to determine if endogenous 2-5A, analogs of 2-5A or other inhibitory factors were induced to cause a down-regulation in the levels of 2-5A-depRNase. IFN, TPA and DiC8 treatment of cells was as described before. An additive effect was observed when an increasing amount of TPA or DiC8 treated cell extract was mixed with IFN treated cell extract (lanes 1, 2, 3).

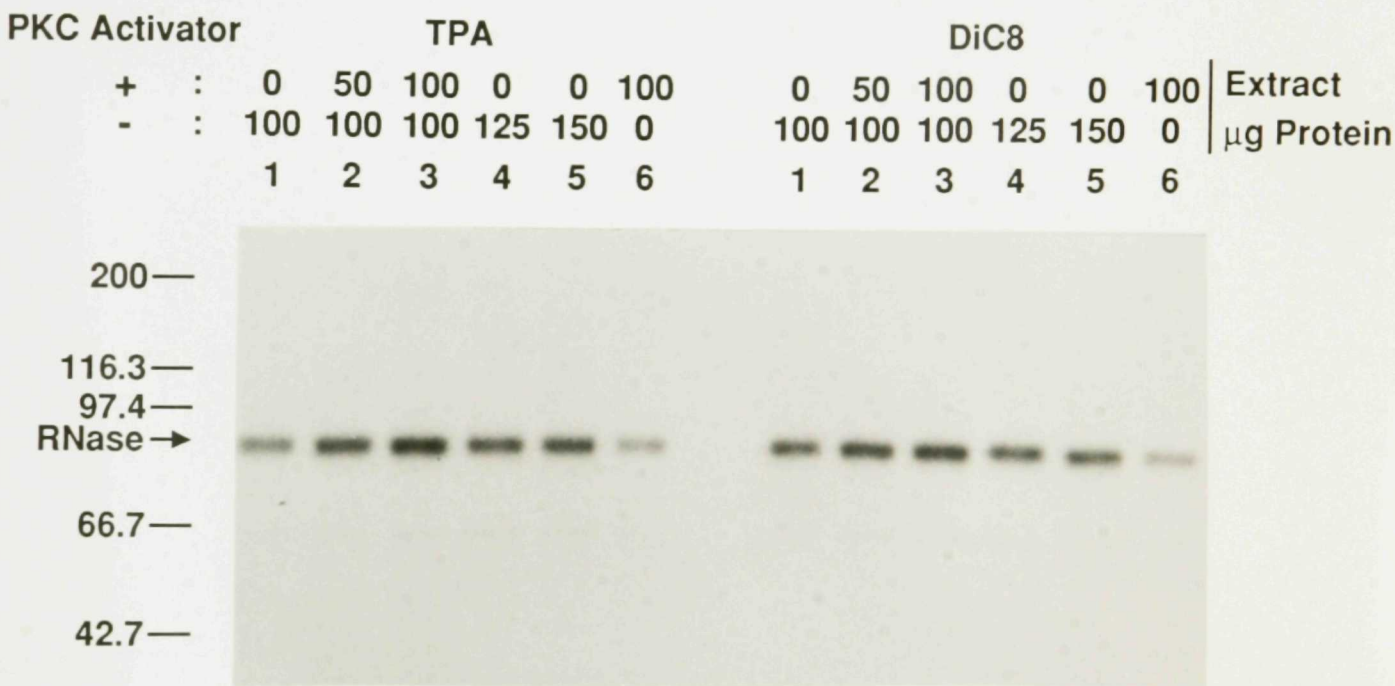

Figure 19.

"Mixing Experiment" to determine if any inhibitory factors were induced in vivo with TPA or DiC8 treatment

Radiobinding assay for detecting 2-5A (figure 20).

2-5A isolated from duplicate samples of JLS-V9R cells; + IFN; TPA alone; TPA + IFN (Table 1), were assayed by the radiobinding method as described in materials and methods. It was important to rule out any endogenous induction of 2-5A or analogs of 2-5A that may compete with the probe for binding to 2-5A-depRNase. The standard curve shown (figure 19), was generated using dilutions of 2-5A (1.2 nM to 250 nM) trimer triphosphate as competitor.

Data obtained (Table 1) showed a small induction of 2-5A in IFN and IFN + TPA treated cells. However, since the induction was about the same, and TPA alone failed to show any significant induction, supports the data from the "Mixing experiments" that down-regulation in the levels of 2-5A-depRNase was not due to induction of any endogenous inhibitory factor.

Figure 20. Radiobinding assay for detecting 2-5A in intact cells.

2-5A isolated from duplicate samples of JLS-V9R cells; + IFN; TPA alone; TPA + IFN, were assayed by the radiobinding method as described in Materials and Methods. The standard curve shown was generated using dilutions of 2-5A (1.2 nM to 250 nM) trimer triphosphate as competitor.

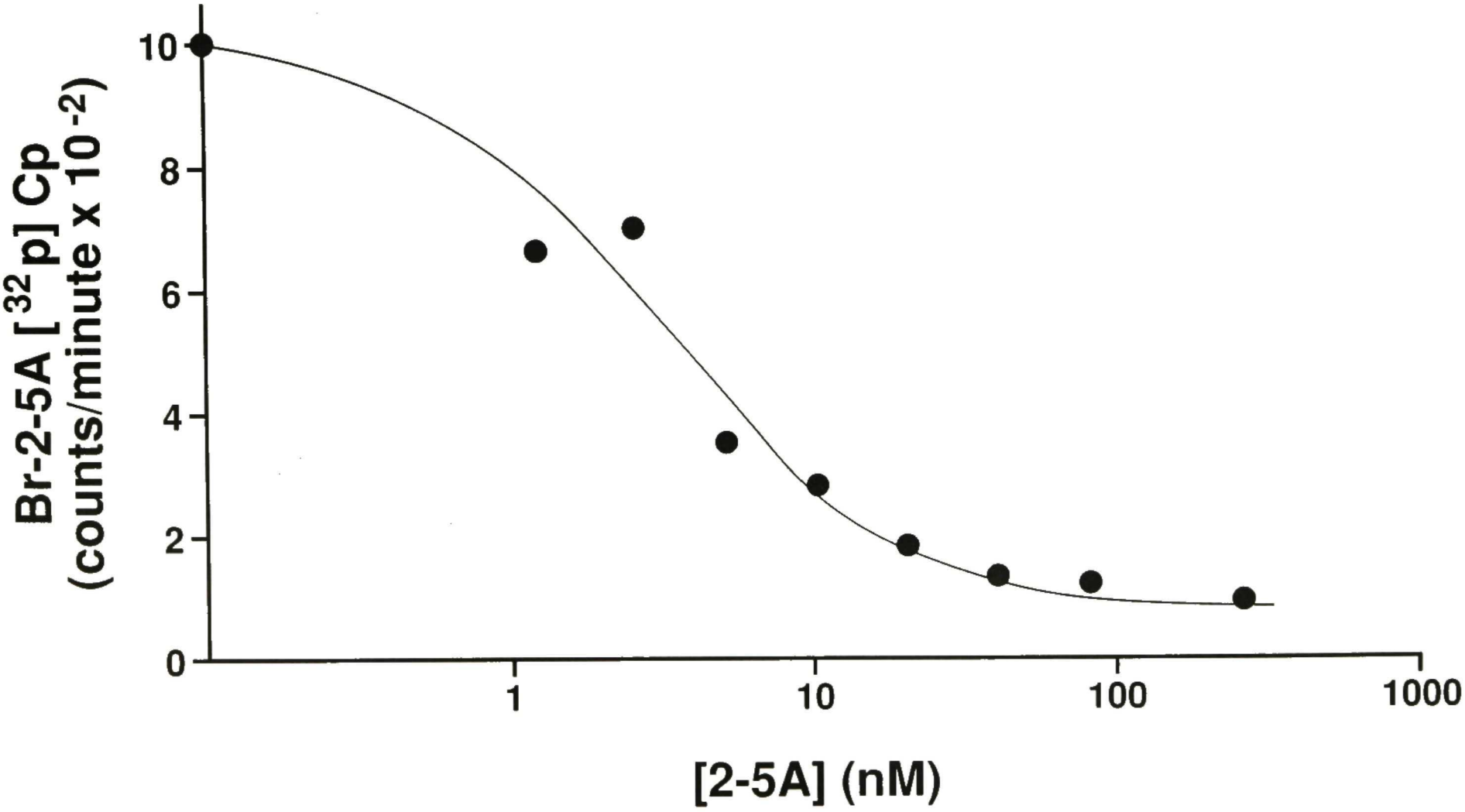

Figure 20.

Radiobinding assay for detecting 2-5A.

Table 1. Results of the Radiobinding assay to determine 2-5A levels in JLS-V9R cells, after IFN and TPA treatment.

Data was obtained using the standard curve shown in figure 20. No significant induction in 2-5A was seen with TPA. IFN treatment however did induce some 2-5A.

Table 1. Results of the Radiobinding assay to determine 2-5A levels in JLS-V9R cells, after IFN and TPA treatment.

Treatment	nM 2-5A Made
Control cells	0.6
IFN	2.7
TPA	0.73
TPA + IFN	3.3

In vitro phosphorylation of partially purified 2-5A-depRNase and purified PKC (figure 21).

To demonstrate a post-translational regulation, possibly at the level of phosphorylation, 2-5A-depRNase was partially purified from mouse liver (as described in materials and methods) and in vitro phosphorylated with purified PKC from rat brain, in the presence of phosphatidylserine, diolein and Ca^{2+}, which are activators of PKC. The reaction shown in lane 1 contained 2-5A-depRNase purified on a 2-5A-sepharose column, PKC and activators. Lane 2 was the same except without PKC. Lane 3 was a control, in which mouse liver extract was cleared with only sepharose (lacking 2-5A), and lane 4 was the same as lane 3, except without PKC.

Phosphorylation of partially purified 2-5A-depRNase could be observed by the addition of [gamma-^{32}P]ATP, purified PKC and activators of PKC, followed by incubation at 30^{o} for 10 min and then subjected to SDS/PAGE. Only lane 1, showed the phosphorylated 2-5A-depRNase band. A second phosphorylated band at 40 KDa is probably a breakdown product of the 77-80 KDa 2-5A-depRNase band. This 40 KDa protein is seen occasionally in mouse liver extracts and after purification of the RNase and can be eliminated or reduced with the addition of a protease inhibitor such as

leupeptin (Krause et al., 1985). Nevertheless, this data shows that the 40 KDa 2-5A binding protein retains its phosphorylation site even after it is subject to proteinase digestion.

In vitro phosphorylation with PKA (figure 22).

Several studies have reported inhibition of cell proliferation by cAMP (Boynton et al., 1983). In JLS-V9R cells, agents that elevate intracellular cAMP levels such as db-cAMP, forskolin and cholera toxin superinduced the 2-5A-depRNase (Fig. 16). The notion that cAMP may be a negative regulator of cell proliferation and findings by Itkes et al.(1985), that elevation of 2-5A synthetase activity and the decrease in 2'-5' phosphodiesterase activity occurs with enhanced cAMP-dependent protein kinase activity in resting NIH 3T3 cells, prompted me to reason that this second messenger may play a role in the regulation of the 2-5A-depRNase during differentiation and growth arrest.

In mammalian cells, cAMP is generated in response to ligand-receptor interaction at the cell surface, transducing that signal by binding to the regulatory subunits (R subunits) of PKA. The PKA holoenzyme is an inactive tetramer of two catalytic subunits (C subunits) and two R subunits, and cAMP binding to the R subunits causes the release of

active C subunits. Many of the biological effects of cAMP are thought to be caused by the phosphorylation of specific substrates by the C subunits (Krebs and Beavo; 1979).

2-5A-sepharose purified 2-5A-depRNase was incubated with PKA and cAMP (1 nM) for 30 min at 30^{o}C as described in Materials and Methods. After incubation, samples were electrophoresed on a 10% SDS-polyacrylamide gel and subjected to autoradiography. PKA + 2-5A-depRNase in the absence (lane 1) or presence of PKA inhibitor (lane 3); PKA alone (lane 2); PKA + Histone (lane 4). Data shows that PKA failed to phosphorylate the partially purified 2-5A-depRNase in vitro.

Figure 21. In vitro phosphorylation of partially purified 2-5A-depRNase with purified PKC.

The phosphorylation assay was performed as described in Materials and Methods. 2-5A-depRNase partially purified on a 2-5A-sepharose column was phosphorylated with purified PKC from rat brain in the presence of PKC activators (lane 1). RNase in the absence of PKC (lane 2). Mouse liver extract was cleared with only sepharose and then phosphorylated with PKC (lane 3).

Figure 21.

In vitro phosphorylation of partially purified 2-5A-depRNase and purified PKC

Figure 22. In vitro phosphorylation of 2-5A-depRNase with cAMP dependent protein kinase (PKA).

Partially purified 2-5A-depRNase was phosphorylated with the catalytic and regulatory subunits of PKA. Assays were performed in 100 ul of 50 uM sodium acetate; 10 uM MgOAc; 5 nM cAMP; 2 uM (20 uCi gamma-^{32}P)ATP, and 3 ug of PKA per ml. RNase + PKA (lane 1); PKA alone (lane 2); RNase + PKA + kinase inhibitor which binds to the catalytic subunit of PKA and possibly inactivates it by dissociating the catalytic subunit from the regulatory subunit (Walsh et al., 1971). The biochemical action, however, of this inhibitor is still not fully undertood. Histone was used as a positive control (lane 4). The two bands, a 45 kDa and 40 kDa, are probably PKA and another contaminating protein that is a substrate for PKA. The 30 kDa band in lane 4 is the phosphorylated histone band. PKA had no effect on the 2-5A-depRNase.

Figure 22.

In vitro phosphorylation of partially purified 2-5A-depRNase and PKA

Inhibition of 2-5A-depRNase phosphorylation with gamma-^{32}p ATP and PKC, after binding with unlabeled 2-5A (figure 23).

Down-regulation of 2-5A-depRNase by activators of PKC and direct demonstration in vitro that it is a phosphoprotein, hinted that a phosphorylation/ dephosphorylation mechanism may in some way modulate its ability to bind 2-5A.

To address this question, partially purified 2-5A-depRNase was first incubated without and with 50, 10 and 5 nM of tetramer 2-5A for 30 min at 0^0C, followed by phosphorylation with PKC as described in Materials and Methods. Quantitation of the results showed that, binding of 2-5A (50 nM) inhibited phosphorylation of the 77-80 kDa 2-5A-depRNase by 2 to 3 fold in three seperate experiments. Autophosphorylation of PKC remained unaltered. However, a similar inhibition in phosphorylation was not observed with the 40 kDa protein. One explanation for this is that proteinase digestion of the 77-80 kDa 2-5A-depRNase may have exposed additional phosphorylation sites which are independent of 2-5A binding. Another possibility that should be considered is that this 40 kDa protein which is a substrate for PKC may not be a 2-5A binding protein.

Covalent binding of the recombinant 2-5A-depRNase with Br-2-5A [^{32}p]Cp, after phosphorylation with PKC and unlabeled ATP (figure 24).

To strengthen the hypothesis that phosphorylation of 2-5A-depRNase inhibits its ability to bind 2-5A, a cDNA encoding the 2-5A-depRNase was in vitro transcribed and then translated in wheat germ extract. The translated product was first phosphorylated with PKC and un-labeled ATP (1 mM), at 30^{o}C for 10 min, and then covalently linked to Br-2-5A [^{32}p]Cp. In this experiment, phosphorylating the 2-5A-depRNase inhibited its subsequent ability to bind the 2-5A probe. The data from this experiment is preliminary and needs further study.

Taken together this data suggests that, a decrease in 2-5A binding in PKC activated JLS-V9R cells may not be due to a decrease in 2-5A-depRNase synthesis, but rather a posttranslational event. Phosphorylation of the 2-5A-depRNase in some way regulates its ability to bind 2-5A.

Figure 23. Phosphorylation of 2-5A-depRNase with (gamma-^{32}P)ATP and PKC, after binding with unlabeled 2-5A.

Partially purified 2-5A-depRNase was first incubated without (lane 2) or with 50, 10, and 5 nM of tetramer 2-5A for 30 min at 0°C (lanes 3, 4 and 5), followed by phosphorylation with PKC as described in Materials and Methods. Autophosphorylation of PKC (lane 1).

Figure 23:

Inhibition of 2-5A-depRNase phosphorylation with gamma-^{32}P ATP and PKC, after binding with unlabeled 2-5A

Figure 24. Covalent binding of recombinant 2-5A-depRNase with Br-2-5A [32p]Cp, after phosphorylation with PKC and unlabeled ATP.

A cDNA encoding the 2-5A-depRNase was in vitro transcribed and then translated in wheat germ extract as described in Materials and Methods. The translated product was first phosphorylated with PKC, activators of PKC and unlabeled ATP (1mM), at 30°C for 10 min (lane 2), or with PKC activators alone (lane 3), and then covalently linked to Br-2-5A [^{32}p]Cp. Control (lane 1), in which the RNase was covalently linked to Br-2-5[^{32}p]Cp in the absence of PKC and PKC activators.

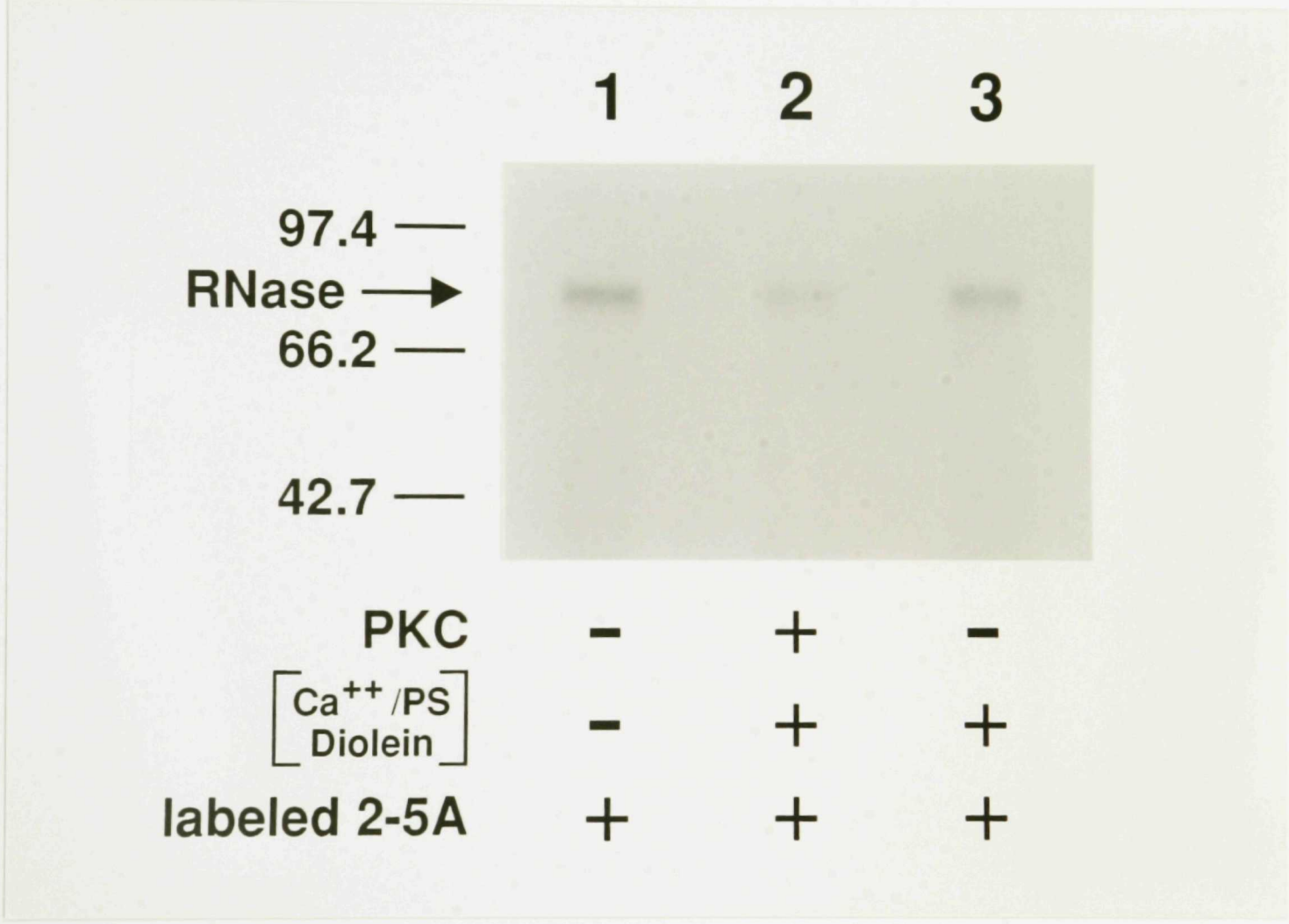

Figure 24:

Covalent binding of the recombinant 2-5A-depRNase with Br-2-5A[^{32}P]Cp, after phosphorylation with PKC and unlabeled ATP.

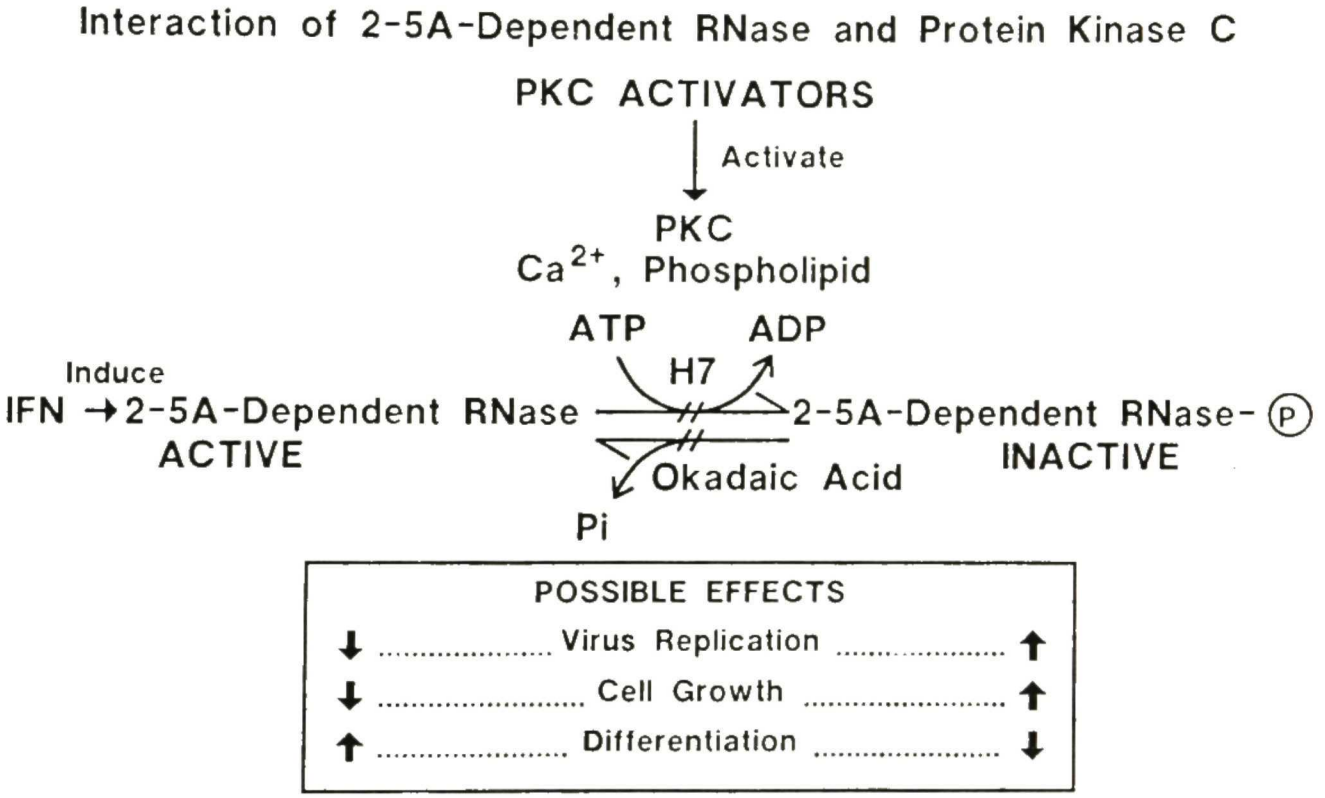

Figure 25:

A working model for the regulation of 2-5A-depRNase

Regulation of 2-5A-depRNase: A working model

The binding of an extracellular stimulatory factor by its cell surface receptor and the transduction of this signal to the interior of the cell is a common mechanism of cell activation in eukaryotic cells. This signal transduction often occurs via the activation of a specific cellular protein kinase which then phosphorylates the receptor and/or regulatory molecules, thereby altering their activity to complete the cellular response to the signal (Kikkawa and Nishizuka 1981).

Exposure of IFN treated JLS-V9R cells to PKC activators, results in the production of diacylglycerols during PI turnover, causing an activation of PKC. Activation of PKC results in a rapid and transient phosphorylation of 2-5A-depRNase, which inhibits its subsequent binding to 2-5A. This differential phosphorylation of 2-5A-depRNase may directly or indirectly contribute to growth suppression, viral infection and differentiation. Based on the present results and the current understanding of the possible growth-suppressive nature of 2-5A-depRNase its cyclic phosphorylation is also suggested. Control of cell proliferation may therefore be exerted at the level of 2-5A-depRNase inactivation.

In conclusion the data presented provide a very clearcut example of the participation of PKC in the regulation of 2-5A-depRNase at the level of its phosphorylation. Moreover, they illustrate the differential binding of 2-5A-depRNase to 2-5A, by a phosphorylation/ dephosphorylation mechanism, which may alter the qualitative nature of the cellular response to extracellular signals during viral infection, growth arrest or differentiation.

DISCUSSION

Cell growth and differentiation within tissues are regulated by a network of paracrine signals transmitted by growth factors and cytokines. Reversible phosphorylation of intracellular proteins, controlled by distinct protein kinases, is closely associated with the process of signal transduction (Cohen, 1982). One major signal transduction pathway depends on PKC, a Ca^{2+}-and phospholipid-dependent enzyme that is activated by diacylglycerol.

My objective was to determine, if activators of PKC, which in several cell lines stimulate growth (Kikkawa, and Nishizuka, 1986), would also lead to qualitative and/or quantitative changes in the 2-5A-depRNase. The levels of 2-5A-depRNase in JLS-V9R cells can vary substantially with cell type and growth conditions. In extracts from IFN treated and confluent cells, higher enzyme levels were observed than from untreated and rapidly proliferating cells (Jacobsen et al., 1983; Floyd-Smith et al., 1982; Krause et al., 1985). Given the close association of both PKC and 2-5A-depRNase with growth status, a possible interaction between these two proteins was hypothesized and investigated.

In these studies, a recently described assay for 2-5A-depRNase (Nolan et al., 1989) was used to evaluate its level in JLS-V9R cells: (a) with IFN-treatment, (b) after short-term exposure to a variety of mitogens known to activate PKC, and (c) after phosphatase inhibition with okadaic acid. 2-5A-depRNase is detected in lysates from several cells by a variety of assays (Knight et al., 1980; Floyd-Smith et al., 1982; Wreschner et al., 1982). However, since 2-5A-depRNase is a very low abundance protein comprising only 0.0002% of the total protein in mouse liver; a concentration of 6 nM (Silverman et al., 1988), its easy detection required the modification of conventional assays to achieve greater sensitivity.

Present findings lead to the following conclusions:

1. The fact that activators of PKC down-regulated 2-5A-depRNase, and inhibitors of PKC such as H-7, superinduced it, as determined by its ability to bind the 2-5A probe, indicated a possible role for PKC in the regulation of the 2-5A-depRNase.

2. Since the down-regulation of the 2-5A-depRNase occurred within 10 to 30 min of exposure to PKC activators, a post-translational regulation, possibly at the level of its phosphorylation was suggested. However, since induction

of the 2-5A-depRNase by IFNs in the JLS-V9R and other cell lines required 8 - 12 hrs, other levels of regulation cannot be ruled out. 2-5A-depRNase may therefore be subject to multiple levels of regulation, depending on the cell type, nutrient conditions and type and duration of the mitogenic stimulus.

3. Treating cells with okadaic acid, a phosphatase inhibitor, resulted in a significant down-regulation of 2-5A-depRNase as determined by its ability to bind Br-2-5A [^{32}p]Cp. Okadaic acid is a potent inhibitor of protein phosphatase types 1(PP1) and 2A(PP2A), which are phosphoserine/threonine phosphatases that reverse the action of many protein kinases including PKC. The net effect of either type of tumor promoter, TPA or okadaic acid is to increase protein phosphorylation of PKC targets.

4. Depleting PKC activity by treating cells with TPA for 16 hrs (Helper et al., 1988), followed by IFN treatment for an additional 16 hrs caused a two-fold superinduction of 2-5A-depRNase (Fig. 13). Because H-7 produced a larger superinduction it may more effectively inhibit PKC than long term TPA treatment.

5. Mixing experiments, using extracts from IFN treated and TPA treated cells, failed to show any endogenous

induction of 2-5A, that may have competed with the probe for binding to 2-5A-depRNase. Assays for 2-5A levels also, failed to show any endogenous induction of 2-5A by TPA. There was however a small induction of 2-5A in IFN-treated cells.

6. To obtain more direct evidence for the involvement of PKC in phorbol-ester-mediated phosphorylation of 2-5A-depRNase, we examined the ability of purified PKC to phosphorylate the partially purified RNase in vitro. Results indicate that 2-5A-depRNase is a substrate for PKC in vitro, and are therefore consistent with the effects of phorbol esters on whole cells being mediated by a direct effect of PKC on 2-5A-depRNase.

Taking these results together, I conclude that it is highly likely the 2-5A-depRNase is a substrate for PKC, and its phosphorylation modulates its ability to bind 2-5A. 2-5A-depRNase may therefore play a key role as a negative regulatory element in cellular growth control. The studies described here have focused on the post-translational regulation of 2-5A-depRNase, and its inactivation/activation by a phosphorylation/dephosphorylation mechanism.

Inhibition of 2-5A binding could occur in two ways.

(i) Phosphorylation of the 2-5A-depRNase could cause an allosteric change in the protein, thereby blocking 2-5A binding.

(ii) The site of phosphorylation and the 2-5A binding could be the same, with one event eliminating the other, depending on which mechanism occurs first.

The mechanism(s) by which IFN and TPA modulate differentiation are not known. The primary site of action for both IFN (Lengyel., 1982) and TPA (Kikkawa, U. and Nishizuka, Y., 1986) appears to be cell membranes. Opposing effects of IFN and TPA on the cell membrane might generate different transmembrane signals and thus either inhibit (IFN) or stimulate (TPA) growth and differentiation. Further investigations of the effects of IFN alone, and in combination with other agents, on cellular growth and differentiation may provide important insights into the mechanisms by which IFN exerts its antitumor effect and also lead to new strategies of combining IFN with other agents to enhance its antigrowth and antitumor effects. Identification of this post-translational regulation of 2-5A-depRNase is of great interest as a potential mediator of the effects of activated PKC in various tissues and cells. Although the mechanisms which govern 2-5A-depRNase function are not

known, we now know that one potential candidate is regulation by phosphorylation. Moreover, phosphorylation of this RNase which inhibits 2-5A from binding, may contribute directly to its inactivation and subsequently to cellular proliferation. Inactivating the functions of the 2-5A-depRNase could allow for the expression of certain proto-oncogenes by extending the half-life of their mRNAs required for growth.

2-5A-depRNase could either:

(i) process mRNA required for growth and differentiation.

(ii) Degrade mRNA for cellular growth factors (including perhaps oncogene products).

The results presented here are in line with the second mechanism noted above. Stimulation of PKC activity which allows for the enhanced expression of a number of oncogenes (Snow et al., 1986; Monroe, J.G., 1988) and also induces the rapid phosphorylation of 2-5A-depRNase. The reduced ability of phosphorylated 2-5A-depRNase to bind 2-5A <u>in vitro</u> and subsequently to be activated may allow for the enhanced expression of these oncogenes.

A major challenge is to provide more convincing evidence that phosphorylation of 2-5A-depRNase actually alters the function of the protein in vivo. The availability of monoclonal antibodies, which unlike 2-5A, may recognize both the phosphorylated and dephosphorylated forms of this protein, should help in the eventual characterizaton of these post-translational level in tissues and cell lines where substantial fluctuations in the level of 2-5A-depRNase (as assayed by binding to radiolabeled probe) is observed as a function of growth. As our understanding increases of how kinases and phosphatases determine phosphorylation states of the 2-5A-depRNase, we may better appreciate how the 2-5A pathway is integrated into the over all physiology of the cell.

Expression of the 2-5A System during the cell cycle.

There are several observations, though some controversial, to support the possibility that 2-5A may be among the agents controlling the rate of cell division. The treatment of lymphocytes with the mitogen Concanavalin A causes a several fold increase in the level of (2',5') PDi without affecting the level of 2-5A synthetase (Kimchi et al., 1981b). The level of (2'-5') PDi is three fold higher in dividing cells than in confluent cells of a monkey cell

line (AGMK). Serum starvation decreases the level of the enzyme, whereas the addition of serum increases it. Finally, the ratio of 2-5A synthetase to (2'-5') PDi, which determines the level of 2-5A, is ten fold lower in fast growing (AGMK) cells than in confluent cells, (arrested in the G_o phase by serum starvation (Kimchi et al., 1979; 1981). However, there is some evidence questioning the role of the 2-5A pathway in growth control. Daudi cells, a human lymphoblastoid cell line is very sensitive to IFNs antigrowth effects (Adams et al., 1975), yet IFN does not enhance the level of 2-5A in these cells (Silverman et al., 1982).

It is now appreciated that the proliferation of cells is governed by an elaborate circuitry that reaches from the surface of the cell to the nucleus (Nishizuka, 1986). In the absence of growth-promoting factors (mitogens), most cells enter a resting state in the G_1 phase of the life cycle and so contain a diploid amount of DNA. Once a cell has begun to replicate its DNA, it continues through the G_2 phase and into mitosis (Sreevalsan., 1984). Once into G_1, a new mitogenic stimulus must be applied before the cell will start another cell cycle. Otherwise, the cell enters a quiescent state, which often leads to differentiation that may create a highly specialized cell(e.g., nerve)unable to initiate a new cycle of growth and cell division. Past

studies have indicated the following characteristics of IFN-induced inhibition of cellular DNA synthesis and division in normal cells:

1. The levels of 2-5A synthetase and 2-5A-depRNase have been reported to vary with the growth condition of cells (Kimchi et al., 1979; Krishnan and Baglioni., 1980; Jacobsen et al., 1983; Floyd-Smith., 1988).

2. The IFN-induced growth inhibition is reflected in an increase in the length of G_1 as well as a reduced rate of cells entering into the S phase (Sreevalsan., 1980).

3. At present it seems logical to assume that the antigrowth effects of IFN are based on its ability to modulate the synthesis of cellular proteins. Conceivably IFNs, acting positively or negatively, can modulate the synthesis of some key proteins during the cells' traverse into the S phase. Based on these and other studies, a speculative model for the relationship between PKC and 2-5A-depRNase during cell cycle in JLS-V9R cells can be proposed.

Orderly cell division depends on the coordination of the events of the cell cycle. In eukaryotes this cordination is produced by the regulation of three cell cycle transitions: entry into mitosis, exit from mitosis, and passage through a point in G1 named "start", that commits

the cell to initiating DNA synthesis(the S phase). In mammalian cells "start" is the point in the cell cycle where cells monitor cell size and nutrient availability (Baserga, R. 1985)

What role does the 2-5A-depRNase play in this cell-cycle transition?

Present work suggests that phosphorylation of 2-5A-depRNase by PKC inhibits its ability to bind 2-5A, which is required for its RNase activity. Thus, translation of mRNA from certain growth factor genes could be facilitated by inhibition of 2-5A-depRNase. That is, inactivation of 2-5A-depRNase by phosphorylation could ensure that certain replication proteins are not present in the G_o or resting state would be expressed, thereby allowing transition from G_o to G_1 to S-phase. The mRNA for growth factors, including proto-oncogenes like c-myc, c-fos and c-myb which have been implicated in growth may fail to be expressed in the presence of adequate levels of 2-5A-depRNase. Complex formation between 2-5A and 2-5A-depRNase may be regulated in part by the cell cycle-dependent phosphorylation and dephosphorylation of 2-5A-depRNase, or the actions of a specific kinase(s), may directly or indirectly, contribute to the efficient dissolution of 2-5A:2-5A-depRNase complex, while that of a specific protein phosphatase may have an

opposite effect. The suggestion that 2-5A-depRNase has a role in regulating the cell-cycle, raises several questions:

1. Are there additional phosphatases and kinases that also regulate the activity of 2-5A-depRNase through phosphorylation?

2. Is there both a cyclical phosphorylation/ dephosphorylation of 2-5A-depRNase as well as cyclical binding and release of 2-5A from 2-5A-RNase?

3. Is there a cyclic circuitry involving the inactivation of 2-5A-depRNase and activation of growth related proteins?

4. Are there other levels of regulation of the 2-5A-depRNase?

Future Studies

Quantitation of phosphorylation of 2-5A-depRNase in vivo may provide insight into the mechanism by which this protein may play a regulatory role in signal transduction, mitosis, cell proliferation and gene regulation.

In Vivo protein phosphorylation

To address this problem, an in vivo double-labeling method (utilizing [^{3}H]-,[^{14}C]-or[^{35}S]-radiolabeled amino acids and [^{32}P]-orthophosphate that allows for quantitation of the amount of specific phosphorylation of a given protein can be used (Capps, G. and Zuniga, M. 1990).

JLS-V9R cells grown in the appropriate media, can be incubated in methionine-free, phosphate-free medium, followed by incubation with [^{35}S]-methionine and [^{32}P]-orthophosphate. Before the cells are harvested, the [^{35}S]-methionine and [^{32}P]-orthophosphate labeled cell suspension can be split, and the phorbol ester (TPA), can be added to one sample. Following lysis, 2-5A-depRNase can be immunoprecipitated from this lysate and be analysed by SDS/PAGE. Two autoradiograms, one specific for ^{35}S, the other for ^{32}P, can be generated from the gel (Capps, G. and Zunigam, M., 1990), allowing for measurement of induction of phosphorylation while controlling for quantity of protein being measured.

Cell Synchronization and Pulse-Chase Experiments.

The data presented here indicate that in the JLS-V9R cells and possibly in other mammalian cells, 2-5A-depRNase

phosphorylation may be tied to specific events in the cell-cycle. In G_o/G_1, also referred to as the resting state (Baserga, 1985), most of the 2-5A-depRNase may exist in an un- or under-phosphorylated state, as determined by its ability to bind 2-5A. In contrast, in S and G_2, during which time most of the DNA synthesis occurs (Baserga, 1985), it may be largely phosphorylated.

To test for the relationship between cell cycle position and the state of 2-5A-depRNase phosphorylation, IFN treated JLS-V9R cells can be growth arrested at G1/S phase with hydroxyurea. Cells can then be pulse-labelled with [^{35}S]methionine. Mitotically arrested JLS-V9R cells can be isolated as described by Shenoy et al.(1989), using Nocodozole (methyl[5-(2thienylcarbonyl)-1H-benzimidizole-2-yl]-carbamate; Jansen). The nonadherent cells are collected by pipetting and centrifugation. After replating,virtually all of these cells selected for viability by virtue of their adherence to the plastic surface in 3hr would be found to be in M phase by light microscopy. This specific population can be extracted and the lysate analysed for phosphorylated and nonphosphorylated forms of 2-5A-depRNase by western blotting. Covalent binding assays using br-2-5A[^{32}p]Cp would also be helpful, by using it with the above extract to determine how the phosphorylation state of the RNase during growth arrest and growth stimulation is regulated.

Subcellular localization of phosphorylated and nonphosphorylated 2-5A-depRNase in tissue culture cells at different times after IFN treatment and growth stimulation would by of particular interest. This can be determined using immunohistologic techniques. In addition, the effects of microinjecting antibodies directed against the 2-5A-depRNase on the ability of cells to synthesize DNA and undergo mitosis can be assessed. Monoclonal or polyclonal antibodies can be used in immunoprecipitation experiments to determine the phosphorylation/dephosphorylation state of the 2-5A-depRNase during cell cycle regulation.

2-5A-depRNase as a suppressor of neoplastic growth.

IFNs are negative regulators of cell growth both in culture and in animals (Taylor-Papadimitriou, 1980). To study the involvement of the 2-5A system in inhibition of tumor growth, the 2-5A-depRNase gene, in both sense or antisense orientation can be introduced into a tumor cell line. This application will permit specific studies to determine the physiological mechanisms by which 2-5A-depRNase impacts regulation of growth, and arrest of tumorigenic cells. Alternatively, a mutant cell line for 2-5A-depRNase and PKC can be used to introduce 2-5A-depRNase gene to study regulation and cell growth.

Does inhibition of cell growth result from the specific cleavage of growth factor mRNA's by 2-5A-depRNase?

Degradation of several oncogene mRNAs is mediated by a repeated hexanucleotide sequence, AUUUA found in their 3'-untranslated regions. Since the dinucleotides UU and UA are favored sites of cleavage by 2-5A-depRNase, the 2-5A system could regulate cell growth by degrading mRNAs of growth factors and oncogenes. Meijlink et al., (1985), have reported that in some proto-oncogenes, including c-myc and c-fos, deletion of the untranslated A-T rich sequence of confers transforming activity when assayed in cultured rat fibroblast cells. This observation led to the proposal that the expression of c-fos gene is regulated post-transcriptionally and that a novel regulation involves the 3' interacting noncoding region. Specific AUUUA rich sequences promoting RNA decay has been identified at the 3' noncoding region of the transiently expressed mRNA protooncogenes c-fos, c-myc, c-myb, c-sis, and cytokines (Brawerman, 1987, Cell, 48, 5-6). Such a sequence has not been identified in the 3' noncoding region of stable mRNA such as globin mRNA. Shaw and Kamen (1986) have shown that insertion of AUUUA rich sequences into rabbit beta-globin gene, destabilized the globin transcript, but the manner in which the AUUUA region promotes RNA decay was not demonstrated. It is possible this AUUUA sequence motif is

cleaved by 2-5A-depRNase, as it has been shown to degrade RNA predominantly at UA and UU sequences (Wreschner et al., 1981; and Floyd-Smith et al., 1981).

A second line of evidence comes from the work of Shaw and Kamen (1986), who have shown that activation of PKC allows for the expression of certain proto-oncogenes. mRNA for granulocyte-monocyte colony stimulating factor (GM-CSF) was reported to be unstable (a half-life of < 30 min) after induction of human T lymphoblast cells with phytohemagglutinin, whereas the GM-CSF mRNA was stable after treatment of the cells with PKC activator TPA. The increased stability of GM-CSF mRNA induced by TPA as opposed to phytahemagglutinin, may be due to the fact that TPA activates PKC, which in turn phosphorylates the 2-5A-depRNase. Phosphorylation of 2-5A-depRNase inhibits its binding to 2-5A, possibly reducing degradation of GM-CSF mRNA.

The potential role played by 2-5A-depRNase in this selective degradation of transiently expressed messengers with such AUUUA sequences is experimentally approachable using the recombinant wild type 2-5A-RNase. Expression vectors for c-fos, which has the AUUUA sequence in the 3' UTR, can be constructed, with and without deletions in that region. Co-transfection of the c-fos plasmid DNA and the 2-

5A-depRNase cDNA can be attempted in the JLS-V9R cells, and expression of the c-fos mRNA can be monitored by northern analysis. Since short term treatment of JLS-V9R cells with PKC activators caused a down regulation in the 2-5A-depRNase, it will be interesting to examine if 2-5A-depRNase phosphorylation can negatively modulate the pathway of mRNA degradation involving the AU sequence element. Perhaps the transient increase in expression of certain proto-oncogenes, initiated by a signal received at the cell surface , may result in part from a temporary block in the activation of 2-5A-depRNase pathway by a phosphorylation/dephosphorylation mechanism.

Bibliography

Adams, A., Strander, H. and Cantell, K. (1975). Sensitivity of the Epstein-Barr virus transformed human lymphoid cell lines to IFN. J. Gen. Virol. 28, 207-217

Aguet, M. (1980). High-affinity binding of ^{125}I-labelled mouse interferon to a specific cell surface receptor. Nature, 284, 459-461

Aguet, M., Grobke, M., and Dreiding, P. (1984). Various human interferon-α subclasses cross-react with common receptors: their binding affinities correlate with their specific biological activities. Virology 132, 211-216

Aguet, M, and Mogensen, K.E. (1983). Interferon receptors. In interferon 5, 1. Gresser, ed. (New York: Academic Press), pp. 1-22

Aguet, M., Dembic, Z., and Merlin, G. (1988). Molecular cloning and expression of the human interferon-gamma receptor. Cell 55, 273-280

Allen, P.T., Schellekens, H., Van Griensen, L., and Billiau, A.J. (1976), J. Gen Virol, 31: 429-435

Apostolov, K., Barker, W. (1981). The effects of Interferon on the fatty acids in uninfected cells. FEBS Lett. 126, 261-264

Ashendel, C.L. (1985). The phorbol ester receptor: a phospholipid regulated protein kinase. Biochim. Biophys. Acta. 822: 219-242

Baglioni, C., Minks, M.A. and Maroney, P.A. (1978). Interferon action may be mediated by activation of a nuclease by pppA2'p5'A2'p5'A. Nature, 279, 684-686

Baserga, R. 1985. The biology of cell reproduction, Harvard University Press, Cambridge, Mass.

Bialojan, C. and Takai, A. (1988). Inhibitory effects of a marine-sponge toxin okadaic acid, on protein phosphatases. Biochem. J. 256, 283-290

Bradford, M. (1976) Anal. Biochem. 72, 248-254

Brawerman, G (1987). Determinants of mRNA stability. cell, 48, 5-6

Brown, R.E., Cayley, P.J., and Kerr, I.M. (1981), In Methods in Enzymology (Pestka, S., ed), Vol. 79,pp.208-216 (Academic

press, New York)

Burn, P. (1988). Amphitropic proteins: a new class of membrane proteins. Trends Biochem. Sci. 13: 79-83

Cardellina, J.H., Marner, F.J. and Moore, R.E. Seaweed dermatitis: structure of lynbyatoxin A. Science, (1979) 204: 193- 195

Castagna, M., Takai,Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982). Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257, 7847-7851

Cayley, P.J., White, R.F., Antoniw, J.F., Walesby, N.J. and Kerr, I.M. (1982). Distribution of the ppp(A2'p)$_n$A-binding protein and interferon-related enzymes in animals, plants and lower animal organisms. Biochem. Biophys. Res. Comm. 108, 1243-1250

Chandrabose, K., Cuatrecasas, P., Pottathil, R. 1981. Changes in fatty acyl chains of phospholipids induced by interferon in mouse sarcoma S-180 cells. Biochem. Biophys. Res. Commun. 98: 661-68

Chebath J., Benech P., Hovanessian A., Galabru J., Revel M. (1987a) Four different forms of interferon-induced 2'5'-oligo(A) synthetase identified by immunoblotting in human cells. J Biol Chem 262: 3852-3857

Chebath J., Benech P., Revel M., Vigneron M (1987b). Constitutive expression of (2'5')oligo A synthetase confers resistance to picornavirus infection. Nature. 330, 587-588

Chitayat, S.D., Nissim, A., Coccia, C., Chebath, J. and Salzberg, S. (1987). 2-5A synthetase gene expression in normal and MSV-transformed NIH/3T3 cells. J. Interferon Res. 7, 730

Clemens, M., and Mcnurlan, M. (1985). Biochem J. 226: 345-360

Cohen, B., Peretz, D., Vaiman, D., Benech, P., and Chebath, J, (1988). Enhancer-like interferon responsive sequences of the human and murine (2'-5') oligoadenylate synthetase gene promoters. EMBO J. 7, 1411-1419

Cohen, P. (1989). The structure and regulation of protein phosphatases. Annu. Rev. Biochem. 58, 453-508

Cope, F.O., Boutwell, R.K. Retnoids: New Trends in Research and Therapy. Retnoid symposium, Geneva 1984, Editor J.H. Saurat, Geneva

Coughlin, S.R., Lee, W. M. F., Williams, P.W., Giels, G. M., and Williams, L.T. (1985). C-myc gene expression is stimulated by agents that activate protein kinase C and does not account for the mitogenic effect of PDGF. Cell 43, 243-251

Courturier, A., Bazgar, S., Castagna, M. (1984). Further characterization of tumor-promoter-mediated activation of protein kinase C. Biochem. Biophys. Res. Commun. 121:448-455

Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E.,Westerfield, M.D., Francke, U and Ullrich, A. (1986). Multiple, distinct forms of Bovine and Human protein kinase C suggest diversity in cellular signalling pathways. Science. 233:859

Datta, P. and Natraj, C. V. (1980). Fibroblast growth regulatory factor inhibits DNA synthesis in Balb/c 3T3 cells by arresting in G_1. Exp. Cell Res. 125, 431-439

Diomond, L., O'Brien, T.G. & Rovera, G. (1977). Inhibition of adipose conversion of 3T3 fibroblast by tumor promoters.

Nature (London) 269, 247-249

Eid, P., Bandu, M.T., Uze, G., Mogensen, K.E. (1988). Interferon removes its own receptors as its blocks the division of Daudi cells. Eur. J. Biochem. 171, 675-682

Etienne-Smekens, M., Goldstein, J., Ooms, H. and Dumont, J. (1983) Eur. J. Biochem. 130, 269-273

Fisher, S.K., Van Rooijen, L.A.A., Agranoff, B.W. (1984). Renewed interest in the polyphosphoinositides. Trends Biochem. Sci. 9: 53-56

Floyd-Smith, G., Yoshie, O. and Lengyel, P. (1981) Covalent linkage of (2'-5')pppApApA(^{32}P)pCp to (2'-5')$(A)_n$-dependent ribonuclease in cell extracts by ultraviolet irradiation. J. Biol. Chem. 257, 8584-8587

Floyd-Smith, G., Slattery, E. and Lengyel, P. (1981a). Interferon action: RNA cleavage pattern of a (2'-5')oligoadenylate-dependent endonuclease. Science 212, 1030-1032

Floyd-Smith, G. (1988). (2'-5')A_n-Dependent Endoribonuclease: Enzyme Levels Are Regulated by IFNβ, IFN gamma, and Cell Culture Conditions. Journal of Cellular

Biochemistry. 38: 13-21

Friedman-Einat, M., Revel, M. and Kimchi, A. (1982) Mol. cell. Biol. 2, 1472-1480

Gilles vze, Georges Lutfalla, and Ion Gresser. (1990), Genetic transfer of a Functional Human Interferon α Receptor into mouse cells: cloning and Expression of its cDNA. Cell, vol 60, 225-2344

Gresser, I. and Trovey, M.G. (1978). Antitumor effects of interferon. Biochem. Biophys. Acta. 516, 231-247

Haavik, J., Schelling, D.L., Campbell, D.G., Anderson, K.K., Flatmark, T. and Cohen, P. (1989). Effects of tumor promoter okadaic acid on intracellular protein phosphorylation and metabolism. FEBS Lett. 251, 36-42

Hannigan, G., Williams, B.R.G. (1986). Transcriptional regulation of interferon-responsive genes is closely linked to interferon receptor occupancy. EMBO J. 5, 1607-1613

Hawthorne, J.N. (1982). Is phosphotidylinositol now out of the calcium gate? Nature (London) 295, 281-282

Haystead, T.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P. and Hardie, D.G. (1989) Nature 337, 78-81

Helper, J.R., Earp, S.H., and Harden, K. (1988). Long term phorbol ester treatment down-regulates protein kinase C and sensitizes the phosphoinositide signaling pathway to hormone and growth factor stimulation. J.Biol. Chem. 263, 7610-7619

Hersh, C.L., Brown, R.E., Roberts, W.K., Swyryd, E.A., Kerr, I.M., and Stark, G.R. (1984). Simian virus 40-infected, interferon-treated cells contain 2',5'-oligoadenylates which do not activate cleavage of RNA. J. Biol. Chem. 259, 1731-1737

Hidaka, H., Inagaki, M., Kawamoto, S., Sasaki, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase C. Biochemistry 23, 5036-5041

Hokin, M.R. and Hokin, L.E. (1953). Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreatic slices. J. Biol. Chem. 203, 967-977

Hovanessian, A.G., Brown, R.E. and Kerr, I.M. (1977).

Synthesis of a low molecular weight inhibitor of protein synthesis with enzyme from interferon treated cells. Nature 268, 537-540

Hovanessian, A.G., Laurent, A.G., Chebath, J., Galabru, J., Robert, N., and Svab. (1987). Identification of 69-Kd and 100-Kd forms of 2-5A synthetase in interferon-treated human cells by specific monoclonal antibodies. EMBO Vol 6 no 5 pp 1273-1280

Hovanessian, A.G., Svab, J., Marie, I., Robert, N., Chamaret, S, and Laurent, A.G. (1988). Characterization of 69-and 100-Kd forms of 2-5A from interferon-treated human cells. J. Biol. Chem. 263, 4945-4948

Huberman, E. & Callaham, M.F. (1979). Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents. Proc. Natl. Acad. Sci. USA 76, 1293-1297

Hunter, T., Ling, N., Cooper, J.A. (1984). Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311: 480-83

Ichii, Y., Fukunaga, R., Shiojiri, S., and Sokawa, Y, (1986). Mouse 2-5A synthetase cDNA: nucleotide sequence and comparison to human 2-5A synthetase. Nucleic Acids Res. 14, 10117

Inglot, A. The biochemical concept of interferon. Arch Virol (1983). 76, 1-13

Irvin, R.G and Dawson, R.M.C. (1979) Biochem. Biophys. Res. Commun. (1979). Transfer of Arachadonic acid between phospholipids in Rat liver microsomes. 91, 1399-1405

Iverson, O.H. (1981) The chalones. In: Baserga, R. (ed), Tissue Growth Factors, Handbook of Experimental Pharmacology, Vol. 57, Springer-Verlag, New York, pp. 491-550

Jacobsen, Helmut., Krause, D., Friedman, R.M., and Silverman, R.H. (1983). Induction of ppp(A2'p)$_n$A- dependent RNase in murine JLS-V9R cells during growth inhibition. Proc. Natl. Acad. Sci. USA, 80, 4954

Jetten, A., and De Luca, L. (1982). Studies on antagonistic actions of TPA and retinoic acid. Carcinogenisis (Lond), 7: 513-520

Johnston, M.I. and Hearl, W.G. (1987). Purification and characterization of a 2'-phosphodiesterase from bovine spleen. J. Biol. Chem. 262, 8377-8382

Johnston, M.I., Torrence, P.F. Interferon vol. 3. Mechanism of production and action. (1984) Elsevier Science Publishers B.V

Kahana, C., Yakobson, E., Revel, M., Groner, Y., (1981). Increased methylation of RNA in SV40-Infected, Interferon-treated cells. Virology 112:109-118

Keay, A. and Grossberg, S.E. (1980). Interferon inhibits the conversion of 3T3-L1 mouse fibroblasts into adipocytes. Proc. Nat. Acad. Sci. USA 77, 4099-4103

Kikkawa, U., Nishizuka, Y. (1986). The role of protein kinase C in transmembrane signalling. Ann. Rev. Cell Biol 2, 149-178

Kimchi, A., Shure, H., Revel, M., (1979). Regulation of lymphocyte mitogenesis by (2'-5')oligoadenylate. Nature 282:849-51

Kimchi, A., Shure, H. and Revel, M., (1981). Anti-mitogenic function of interferon induced (2'-

5')oligo(adenylate) and growth-related variations in enzymes that synthesize and degrade this oligonucleotide. Eur. J. Biochem. 114, 5-10

Kimchi, A., Shure, H., Lapidot, S., Rapoport, S., Panet, A. and Revel, M. (1981). Antimitogenic effects of interferon and (2'-5')-oligoadenylate in synchronized 3T3 fibroblasts. FEBS Letts., 134, 212

Kishimoto,A.,Takai,Y., Mori,T.,Kikkawa,U.and Nishizuka,Y. (1980). Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphotidylinositol turnover. J. Biol. Chem. J. Biol. Chem., 255, 2273-2276

Knight, E. (1976). Antiviral and cell growth inhibitory activities reside in the same glycoprotein of human fibroblast interferon. Nature 262, 302-303

Knight, E. and Korant, B.D. (1977). A cell surface alteration in mouse L cells induced by interferon. Biochem. Biophys. Res. Commun. 74, 707-713

Knight, M., Cayley, P.J., Silverman, R.H., Wreschner, D.H., Gilbert, C.S., Brown, R.E. and Kerr, I.M. (1980). Radioimmune, radiobinding and HPLC analysis of 2-5A and

related oligonucleotides from intact cells. Nature 288, 189- 192

Knopf, J.L., Lee, M.H., Sultzman, L.A., Kriz, R.W., Loomis, C.R., Mewick, R.M and Bell, M.R. Cell (1986) 48:491

Krause, D., Silverman, R.H., Jacobsen, H., Leisy, S.A., of $ppp(A2'p)_nA$-dependent RNase levels during interferon treatment and cell differentiation. Eur. J. Biochem. 146, 611-618

Krebs, E. G. & Beavo, J. A. (1979) annu. Rev. Biochem. 48, 923-959

Krishnan, I. and Baglioni, C. (1980). Increased levels of (2'-5')oligo(A) activity in human lymphoblastoid cells treated with glucocorticoids. Proc. Natl. Acad. Sci. USA, 77, 6506-6510

Krishnan, I. and Baglioni, C. (1981). Elevated levels of (2'-5')oligoadenylic acid polymerase activity in growth-arrested human lymphoblastoid Namalva cells. Mol. cell. Biol. 1, 932-938

Kuo, J.F., Anderson, R.G.G., Wise, B. C., Mackerlova, L., Solomonsson, I., Nancy, L., Brackett., Norio Katoh., Memoni, Shoji., and Robert W. Wrenn. (1980). Calcium-dependent protein kinase: widespread occurence in various tissues and phyla of the animal kingdom and comparison of effects of phospholipid, calmodulin and trifluoperazine. Proc. Natl. Acad. Sci. U.S.A. 77, 7039

Laemlli, U.K. (1970) Nature. 227, 680-685

Laurence, L., Marti, J., Roux, D. and Cailla, H. (1984). Immunological evidence for in vivo occurence of (2'-5') adenyladenosine oligonucleotides in eucaryotes and procaryotes. Proc. Nat. Acad. Sci. USA 81, 2322-2326

Liberman, M.S., Raben, D. and Glaser, L. (1981). Cell surface-associated growth inhibitory proteins. Exp. Cell Res. 133, 413-420

Lin, S.L., Gatber, F.A., Wang, E. Reduced synthesis of pp60 src and expression of the transformation related phenotype in interferon treated Rous sarcoma virus transformed rat cells

Maor, G., Sslzberg, S., Silbermann, M., (1990). The activity of 2,5-oligoadenylate synthetase, an interferon-induced

enzyme, is coupled to the differentiation state of mouse condylar cartilage. Differentiation 44:18-24

Martin, G.R., (1975). Teratocarcinomas as a model system for the study of Embryogenesis and neoplasia. Cell 5: 229

May, W.S., Jr., N. Sahyoun, M. Wolf, and P. Cuatrecasas. (1985). Role of intracellular calcium mobilization in the regulation of protein kinase C-mediated membrane processes. Nature. 317: 549-551

Meijlink et al., (1985). Proc. Natl. Acad. Sci. 82, 4987-4991

Merlin, G., Falcoff, E., and Aguet, M. (1985). ^{125}I-labelled human interferons alpha, beta and gamma: comparative receptor binding data. J. Gen. Virol. 66, 1149-1152

Miao, R.M., Fieldsteel, A. H. & Fodge, D. W. (1978). Opposing effects of tumor promoters on erytheroid differentiation. Nature (London) 274, 271-272

Michell, R.H.,(1975). Inositol phospholipids and cell surface receptor function. Biochim. Biophys. Acta 415,81

Minakuchi, R., Takai, Y., Nishizuka, Y. (1981). Widespread occurence of calcium-activated, phospholipid-dependent protein kinase in mammalian tissues. J. Biochem. (Tokyo) 89, 1651

Minks, M.A., Benvin, S., Maroney, P.A. and Baglioni, C. (1979). Metabolic stability of 2'5' oligo(A) and activity of 2'5' oligo(A)-dependent endonuclease in extracts of interferon treated and control Hela cells. Nucleic Acids Res. 6, 767-780

Mogensen, K.E., and Bandu, M.T (1983). Kinetic evidence for an activation step following binding of human interferon α2 to the membrane receptors of Daudi cells. Eur. J. Biochem. 134, 355-364

Mogensen, K. E., Uze, G., and Eid, P. (1989). The cellular receptor of the alpha-beta interferons. Experientia 45, 500-508

Nilsen, T.W., Wood, D.L. and Baglioni, C. (1981). 2',5'-oligo(A)-activated endoribonuclease. Tissue distribution and characterization with a binding assay. J. Biol. Chem. 256, 10751-10754

Nielsen, T.W., Maroney, P.A. and Baglioni, C. (1982). Synthesis of (2'5')oligo(A) and activation of an endoribonuclease in interferon-treated HeLa cells infected with reovirus. J. Virol. 42, 1039-1045.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. Science 233, 305-312

Nolan-Sorden, N.L., Lesiak, K., Bayard, B., Torrence, P.F. & Silverman, R.H. (1990). Photochemical crosslinking in oligonucleotide-protein complexes between a bromine-substituted 2-5A analog and 2-5A-dependent RNase by UV lamp or laser. Anal. Biochem. 184, 298-304

Parker, P.J., Coussens, L., Notty, N., Rhee, L., Young, S., Chen, E., Stable, S., Waterfield, M.D and Ullrich, A. Science (1986). The complete primary structure of protein kinase C - the major phorbol ester receptor. 233:853

Person, D.A., Wilkison, W.O, Bell, R.M., and Finn, O.J. (1988). Altered growth regulation and enhanced tumorigenicity of NIH 3T3 fibroblasts transfected with protein kinase C-1 cDNA. Cell 52, 447-458

Rashidbaigi, A., Langer, J.A., Jung, V., Jones, C., Morse, H.G., Tischfeld, J.A., Trill, J.S., Kung, A., and Pestka, S

(1986). The gene for the human immune interferon receptor is located on chromosome 6. Proc. Natl. Acad. Sci. U.S.A 83, 384-388

Reid, T.R., Hersh, C.L., Kerr, I.M., Stark, G. R. Analysis of 2'5'-Oligoadenylates in cells and tissues. (1983). Analytical Biochemistry 135, 000-007

Riabowol, K., Draetta, G., Brizuela, L., Dale V, and Beach, D. (1989), Cell, Vol 57, 393-401

Roberts, R.M. (1989). A novel group of interferons associated with the early ovine and bovine embryo. J. Interferon research 9:4605-4612

Rovera, G., O'Brien, T.B. & Diamond, L. (1977). Tumor promoters inhibit spontaneous differentiation of friend erytheroleukemia cells in culture. Proc. Natl. Acad. Sci. USA 74, 2894-2898

Rovera, G., O'Brien, T.G. & Diomond, L. (1979). Induction of differentiation in human promyelocytic leukemia cells by tumor promoters. Science 204, 868- 870

Rossi, G.B., Dolei, A., Cioe, L., Benedetto, A., Matarese, G.P. and Belardelli, F. (1977). Inhibition of

transcription and translation of globin messenger RNA in dimethyl-sulfoxide-stimulated Friend erthroleukemia cells, treated with interferon. Proc. Nat. Acad. Sci. USA 74, 2036-2040

Rubinstein, M., Orchansky, P: In Fasman, G.D (ed): " Critical reviews in biochemistry," vol 21, Boca Raton, Florida: CRC Press, 1986, 249-279

Saunders, M., Gewert, D., Castelino, M., Rutherford, M., Flenniken, A., Willard, H. and Williams, B.R.G. (1985). "Characterization of the small 2-5A synthetase gene in human and mouse cells." The 2-5A system: Molecular and Clinical Aspects of the Interferon-Regulated 2-5A System. (New York, Alan R. Liss) eds. R.H. Silverman and B.R.G Williams pp 163-174

Schwabe, M., Princler, G.L., and Faltynek, C.R.(1988). Characterization of the human type 1 interferon receptor by ligand blotting. Eur. J. Immunol. 18, 2009-2014

Schmidt, A., Chernajovsky, V., Schulman, L., Federman, P., Berissi, H. and Revel M. (1979). An interferon induced phosphodiesterase degrading (2'-5') oligoadenylate and the C-C-A terminus of tRNA. Proc. Nat. Acad. Sci. USA 76, 4788- 4792

Sen G., Lebleu, B., Brown, G.E., Rebello, M.A., Furuichi, Y., Morgan, M., Shatkin, A., Lengyel, P. (1975). Biochem. Biophys. Res. Commun. 65: 427-34

Shibata, S., Ishida, Y., Kitano, H., Ohizumi, Y., Habon, J., Tsukitani, Y. and Kikuchi H. (1982). J. Pharmacol. Exp. Ther. 223, 135-143

Silverman, R.H., Cayley, P.J., Knight, M., Gilbert, C.S. and Kerr, I.M. (1982a) Control of the ppp(A2'p)$_n$A system in Hela cells. Effects of interferon on virus infection. Eur. J. Biochem. 124, 131-138

Silverman, R.H., Watling, D., Balkwill, F.R., Trowsdale, J. and Kerr, I.M. (1982b) The ppp(A2')$_n$A and protein kinase systems in wild-type and interferon-resistant Daudi cells. Eur. J. Biochem. 126, 333-341

Silverman, R.H., Skehel, J.J., James, T.C., Wreschner, D.H. and Kerr, I.M. (1983). Ribosomal RNA cleavage as an index of ppp(A2'p)$_n$A activity in interferon-treated encephalomyocarditis virus-infected cells. J. Virol. 46, 1051-1055

Silverman, R.H., Analytical Biochemistry (1985). Functional Analysis of 2-5A-Dependent RNase and 2-5A, using 2',5'-

Oligoadenylate-Cellulose. 144, 450-460

Silverman, R, and Krause, D. (1986). In, INTERFERONS - A PRACTICAL APPROACH (Morris, A.G. and Clemens, M.J., eds.) IRL Press, Oxford - Wahington, D.C. 1986 In Press.

Silverman, R.H., Jung, D.D., Nolan, N.L., Dieffenbach, C.W., Kedar, V.P., and SenGupta, D.N. (1988). Purification and analysis of murine 2-5A-dependent RNase. J. Biol. Chem. 263, 7336-7341

Slattery, E., Ghosh, N., Samanta, H. and Lengyel, P. (1979). Interferon, double-stranded RNA and RNA degradation: activation of an endonuclease by (2'-5')A_n. Proc. Nat. Acad. Sci. USA 76, 4778-4782

Sporn, M., and Roberts, A. (1983). Role of retinoids in differentiation and carcinogenisis. Cancer Res., 43: 3034-3040

Sreevalsan, T., Rozengurt, E., Taylor-papadimitriou, J. and Burchell, J. (1980). Differential effects of Interferon on DNA synthesis, 2-deoxyglucose uptake and ornithine decarboxylase activity in 3T3 cells stimulated by polypeptide growth factors and tumor promoters. J. Cell. physiol. 104, 1-9

Sreevalsan, T. (1984). Effects of interferon on cell physiology. Interferon volume 3. Mechanism of production and action. Elsevier publication. 343-387

Stark, G.R., Dower, W.J., Schimke, R.T., Brown, R.E. and Kerr, I.M. (1979). 2-5A Synthetase: assay, distribution and variation with growth or hormone status. Nature, 278, 471

St. Laurent, G., Yoshie, O., Floyd-Smith, G., Samanta, H., Sehgal, P.B. and lengyel, P. (1983). Interferon action: two (2'5')$(A)_n$ synthetases specified by distinct mRNAs in ehrlich Ascites Tumor cells treated with interferon. Cell 33, 95-102

Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, s., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamado, K. and Sugimura, T. (1988) Proc. Natl. Acad. Sci. USA 85, 1768-1771

Sugimora, T. Potent tumor promoters other than phorbol ester and their significance. Gann, (1982) 73: 499-507

Tachibana, K., Scheur, P.J., Tsukitani, Y., Kikuchi, H., Van Eden, D., Clardy, J., Gopichand, Y., and Schmitz, F.J. (1981) J.Am. Chem. Soc. 103, 2469-2471

Takai,Y.,Kishimoto,A., Kikkawa,U.,Mori,T.and Nishizuka,Y (1979). Unsaturated Diacylglycerol as a possible messenger for the activation of calcium- activated, phospholipid dependent protein kinase system. Biochem. Biophys. Res. Commun. 91, 1218-1224

Taylor-Papadimitriou, J. (1980). "Effects of interferons on cell growth and function." In interferon vol. 2. I. Gresser, ed. (New York, Academic Press) pp 13-46

Tovey, M.G., Streuli, M., Gresser, I., Gugenheim, J., Blanchard, B., Guymarho, J., Vignaux, F., and Gigou, M. (1987). Interferon messenger RNA is produced constitutively in the organs of normal individuals. Proc. Natl. Acad. Sci. 84:5038-5042

Vanden Broecke, C., and Pfeffer, L.M. (1988). Characterization of interferon-α binding sites on human cell lines. J. interferon Res. 8, 803-811

Walsh, D., Ashby, C., Gonzalez, C., Calkins, D., Fischer, E, and Krebs, E. (1971). Purification and Characterization of a Protein Inhibitor of Adenosine 3',5'-Monophosphate-dependent protein Kinases. J. Biol. Chem., 246:1977

Whittenberger, B., Raben, D., Lieberman, M.A. and Glaser, L. (1978). Inhibition of growth of 3T3 cells by extract of surface membrane. Proc. Natl. Acad. Sci. U.S.A. 75, 5457-5461

Williams, B.R.G., Kerr, I.M., Gilbert, C.S., White, C.N., and Ball, L.A. (1978a). Synthesis and break-down of pppA2'p5'A2'p5'A and transient inhibition of protein synthesis in extracts from interferon and control cells. Eur. J. Biochem. 92, 455-462

Williams, B.R.G. and Kerr, I.M. (1978b). Inhibition of protein synthesis by 2'-5' linked adenine oligonucleotides in intact cells. Nature, 279, 88-90

Williams, B.R.G., Golger, R.R., Brown, R.E., Gilbert, C.S., and Kerr, I.M. (1979). Natural occurance of 2-5A in interferon-treated EMC virus-infected L cells. Nature (London) 282, 582-586

Wreschner, D.H., McCauley, J.W., Skehel, J.J. and Kerr, I.M. (1981). Interferon action-sequence specificity of the ppp(A2'p)$_n$A-dependent ribonuclease. Nature, 289, 414-417

Wreschner, D.H., Silverman, R.H., James, T.C., Gilbert, C.S. and Kerr, I.M. (1982) Affinity labelling and

characterization of the ppp(A2'p)$_2$A-dependent endoribonuclease from different mammalian sources. Eur. J. Biochem. 124, 261-268

Yamasaki, H., Fibach, E., Nudel, U., Weinstein, I. B., Rifkind, R.A. & Marks, P.A. (1977). Tumor promoters inhibit spontaneous and induced differentiation of murine erythroleukemia cells in culture. Proc. Natl. Acad Sci. USA 74, 3451-3455

Yamanishi, J., Takai, Y., Kaibuchi, K., Sano, K., Castagna, M and Nishizuka, Y. (1983). Synergistic functions of phorbol ester and calcium in serotonin release from human platelets. Biochem. biophys. Res. Commun. 112:778-786

Yoshimi, Takai., Akira, Kishimoto., Yasushi, Iwasa., Yasuhiro, Kawahara., Terutoshi, Mori, and Yasutomi Nishizuka. (1979). J. Biol. Chem. 254, 3692-3695

Zarling, J. M., Eskra, L., Borden, E.C., Horoszeiviez, J. and Carter, W.A. (1979). Activation of human natural killer cells cytotoxic for human leukemia cells by purified interferon. J. Immunology. 123, 63-70