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Characterization of intracellular intermediates in v-Fps-initiated signal transduction

Alexandropoulos, Konstantina, Ph.D.

City University of New York, 1992

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CHARACTERIZATION OF INTRACELLULAR INTERMEDIATES IN v-FPS-INITIATED SIGNAL TRANSDUCTION

by

Konstantina Alexandropoulos

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1992

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Abstract

CHARACTERIZATION OF INTRACELLULAR INTERMEDIATES IN v-FPS INITIATED SIGNAL TRANSDUCTION

by

Konstantina Alexandropoulos

Adviser: Dr. David A. Foster

Fujinami Sarcoma Virus (FSV), encodes a phosphoprotein, v-Fps with a kinase activity that phosphorylates proteins on tyrosine residues. The protein tyrosine kinase (PTK) activity of v-Fps can cause rapid transformation *in vitro* and tumor formation *in vivo*. Activating the PTK activity of v-Fps leads to induction of intracellular signaling pathways through the activation of cytoplasmic signaling molecules. In this study, induction of gene expression through activation of the PTK activity of a temperature sensitive (ts) derivative v-Fps was utilized in order to study v-Fps-induced signaling. Inhibitors, specific for putative signaling molecules, that interfere with induction of transcription were used to implicate these molecules in v-Fps initiated signal transduction. Evidence is presented that v-Fps activates a PKC-dependent pathway mediated through a GTP-binding protein (G-protein). Involvement of a heterotrimeric G-protein was suggested using nonhydrolyzable GTP and GDP analogs. GTPγS induced the expression of a v-Fps responsive reporter gene, and phosphorylation of a PKC substrate. GDPβS blocked induction of both of these phenotypes in response to v-Fps but not in response to phorbol esters. These data are consistent with involvement of a G-Protein functioning upstream of PKC in v-Fps signaling. v-Fps also induces expression of a reporter gene in a PKC-independent manner. Dominant negative inhibitors of c-HaRas and Raf-1 inhibit PKC-independent signals induced by v-Fps, where Ras functions upstream of Raf-1. The dominant inhibitor of Ras also inhibits PKC-mediated signals, and depletion of PKC shows that Ras functions downstream from PKC.

The data presented here suggest that v-Fps can activate at least two distinguishable intracellular signaling pathways, one mediated by a heterotrimeric G-protein and PKC, and one that is sequentially dependent upon Ras and Raf-1 and is PKC-independent. The data also suggest that Ras plays a central role as a transducing molecule in v-Fps-induced signaling, by mediating two distinct intracellular signaling pathways.

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ABBREVIATIONS

ALV - Avian Leukosis Virus

CAT - Chloramphenicol Acetyl Transferase

CEF - Chicken Embryo Fibroblasts

CSF - Colony Stimulating Factor

DG - Diacyl glycerol

EGF - Epidermal Growth Factor

FSV - Fujinami Sarcoma Virus

GAP - GTPase Activating Protein

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

G-protein - GTP binding protein

IP3 - Inositol 1,4,5-triphosphate

MARCKS - Myristoylated-Alanine Rich-C kinase Substrate

NGF - Nerve Growth Factor

PBt2 - Phorbol Dibutyrate

PC12 - Phyochromocytoma Cells 12

PDGF - Platelet Derived Growth Factor

PIP2 - Phosphatidylinositol 4,5-bisphosphate

PKC - Protein Kinase C

PLC - Phospholipase C

PTK - Protein Tyrosine Kinase

RSV - Raous Sarcoma Virus

SAP - Saponin

SH2 - Src Homology Region 2

SRE - Serum Response Element

SRF - Serum Response Factor

TPA - 12-O-tetradecanoyl-13- acetate

TRE - TPA Response Element

INTRODUCTION

Cellular transformation and malignant neoplasms develop through a series of changes that result in abnormal expression or function of specific cellular genes. Genes that have been genetically altered to induce malignant transformation or neoplasms are termed oncogenes. Oncogenes were first identified in acutely transforming retroviruses isolated from tumors of animals that were chronically infected with nontransforming retroviruses. The first example for the occurrence of such event came from chickens chronically infected with the weakly oncogenic avian leukosis virus (ALV), which induced tumors only after long latent periods in infected animals. Occasionally, chicken infected with ALV, developed tumors from which new highly oncogenic viruses could be isolated. This new virus, known as the Rous Sarcoma Virus (RSV) (Rous, 1911) rapidly induced sarcomas in infected chickens and efficiently transformed fibroblasts in culture (general references Weiss et al., 1985, Cooper, 1990).

Isolation of acutely transforming retroviruses from nonpathogenic progenitors suggested the possibility that, the transforming viruses contained genes that were derived from the host cells and were incorporated into the genomes of nontransforming retroviruses to yield new viruses with increased pathogenicity. Supporting this hypothesis was the fact that the size of the RSV genomic RNA was approximately 10 kb where that of ALV was approximately 8.5 kb (Duesberg and Vogt 1970). The isolation of deletion RSV mutants that were lacking the extra RNA were also defective in transforming activity, further substantiated the hypothesis that the extra RNA contained the information responsible for the unique biological activity of the transforming virus. The first experimental evidence in reference to the origin of retroviral oncogenes was presented by Stehelin et al., (1976), who were able to isolate a src-specific probe that hybridized to RSV RNA, but not to RNAs from ALV or transformation defective RSV mutants. The src-specific probe also hybridized

extensively to chicken DNA demonstrating that chickens contained DNA closely related to the src oncogene. Analogous experiments with probes for the oncogenes of other acutely transforming viruses led to the discovery of numerous oncogenes with cellular counterparts, normally found in the genomes of many species.

The gene product encoded by the RSV genome was first identified by Brugge and Erickson (1977), using sera from rabbits bearing RSV-induced tumors. Sera from these rabbits reacted with a 60kd protein from chicken and mammalian cells transformed by RSV, where this protein was not detected in cells transformed with RSV mutants defective for transformation. Identification of the src gene product subsequently provided the tools necessary towards understanding how an oncogene may act. Shortly after the discovery of the src protein, src immunoprecipitates were shown to phosphorylate a substrate *in vitro* in the presence of radiolabeled ATP (Collett and Erickson, 1978). Thus, the src protein was the first oncogene found to be a protein kinase; in addition, src was found to phosphorylate proteins on tyrosine residues (Hunter and Sefton, 1980). This was first demonstrated by Eckhart et al., (1979), who observed tyrosine phosphorylation in T antigen immunoprecipitates of polyoma virus transformed cells, which are now known to contain the cellular src gene product.

The findings presented above established tyrosine phosphorylation resulting from oncogenic protein tyrosine kinase (PTK) activity, as being related to transformation. The importance of phosphorylation of proteins on tyrosine was further substantiated by the discovery of additional oncogene products such as abl (Abelson and Rabstein, 1970), fes/fps, fms, ros and yes, that also were protein-tyrosine kinases involved in neoplastic growth.

The protein tyrosine kinases identified to date, are classified into two major categories, receptor and nonreceptor tyrosine kinases. Both types of protein tyrosine kinases can serve as signal transducers of information within the cell, and respond to

stimuli by increasing their catalytic activity and transferring information to target molecules by phosphorylation. Receptor and non-receptor tyrosine kinases have been implicated in transduction of signals that lead to changes in gene expression, DNA synthesis, and cell proliferation through complex intracellular signaling pathways. The mechanisms leading to the activation of these pathways have not been well characterized; nonetheless, numerous cytoplasmic and nuclear molecules have been implicated as possible intermediates (reviewed by Hanks et al., 1988; Hunter and Cooper, 1985; Yarden and Ullrich, 1988; Cantley et al., 1991).

Protein kinase C (PKC), a serine/threonine protein kinase, has been implicated in a key role in transduction of mitogenic signals. Possible functions for PKC include involvement in secretion and exocytosis, modulation of ion conductance, regulation of receptor interaction with components of the signal transduction apparatus, gene expression and cell proliferation (reviewed by Nishizuka 1986). Activation of PKC is an early event in mitogenesis, resulting from growth factor-stimulated phospholipid hydrolysis that leads to the production of diacylglycerol (DG) which activates PKC. Activation of PKC results in transmission of the signal to the nucleus via activation of transcription factors (jun/fos) (Angel et al., 1987), which then modulate the expression of target genes.

c-Raf-1, also a serine/threonine kinase, has been implicated as a potential signal transducer for tyrosine kinases. c-Raf-1 is the cellular homolog of v-Raf, the transforming gene of the murine sarcoma virus 3611. The protein product of the c-Raf-1 gene is a phosphoprotein of 70-75kd that phosphorylates other proteins on serine and threonine residues (reviewed by Rapp, 1991). The role of Raf-1 in signal transduction has been shown in response to platelet growth factor (PDGF) (Morrison et al., 1988) and other mitogens as well as the src oncogene (Morrison et al., 1988).

Stimulation of cells with mitogens leads to a change in the apparent molecular weight of Raf-1 which is manifested as a mobility shift of the vast majority of Raf-1 molecules to a higher molecular weight. This size change has been shown to be caused by hyperphosphorylation of the protein on serine residues (Morrison et al., 1988), (reviewed by Roberts, 1991). In addition to the mobility shift, the level of kinase activity in Raf-1 immunoprecipitates increases significantly in response to growth factors, phorbol esters and other mitogens (Morrison et al., 1989). Activation of Raf-1 in response to oncogenes and growth factors occurs predominantly on serine residues, although tyrosine phosphorylation of Raf-1 is also detected in PDGF-treated and Src-transformed cells (Morrison et al., 1988). Thus, activation of Raf-1 may occur by either phosphorylation on serine/threonine residues, which can be mediated by PKC, or by direct phosphorylation on tyrosine by receptor and non-receptor tyrosine kinases. These observations suggest a general role for Raf-1 in transducing signals initiated by plasma membrane proteins, from the cell surface to the nucleus.

Mechanisms involved in receptor signaling other than phosphorylation cascades involving kinases, include GTP-binding proteins (G-proteins) which propagate intracellular signals by directly interacting with both receptors and effector molecules (reviewed by Gilman, 1987, and Bourne, 1991). G-proteins implicated in signal transduction comprise two major families: the heterotrimeric class of G-proteins, and the monomeric $p21^{ras}$ gene family. Heterotrimeric G-proteins consist of three subunits α, ß, and τ, and their activity is regulated by nucleotide binding where GTP activates and GDP inhibits protein function. In the resting state G-proteins are bound to a receptor. Ligand binding to the receptor results in exchange of bound GDP for free GTP on the α subunit. Thus activated, α subunits dissociate from the ßτ subunits and the receptor and interact with target molecules to lead to production of second messengers. Second messenger systems modulated by G-protein coupled receptors

include (reviewed by Gilman 1987): 1) the adenylate cyclase system that is regulated by hormone receptors coupled to Gs which leads to production of cAMP, and Gi which inhibits production of cAMP. cAMP in turn activates cAMP-dependent protein kinase which then modulates cellular responses; 2) The rhodopsin-transducin system, which functions in the retina to couple the photoreceptor rhodopsin to the cGMP system. Photoactivation of rhodopsin activates transducin which stimulates cGMP phosphodiesterase to lead to increases in cGMP concentration; 3) the G-protein-inositol phospholipid-coupled system where hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C, leads to production of intracellular messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG). Activation of the phospholipid pathway and its second messengers results in cell proliferation; 4) G-protein-Ca^{++} and K^{+} channels-coupled systems where G-proteins have been shown to regulate ion channel conductivity.

The monomeric $p21^{ras}$ family consists of related monomeric proteins that are localized to the inner face of the plasma membrane and bind guanine nucleotides with high affinity (reviewed by Bourne, 1991). The GTP binding activity of the Ras proteins, first suggested that they might function analogously to the heterotrimeric protein in coupling receptors to intracellular second messengers. The analogy between Ras and heterotrimeric G-proteins was further substantiated with evidence showing that Ras possessed intrinsic GTPase activity, suggesting that Ras proteins alternated between the GTP (active) and GDP (inactive) forms (Bourne, 1991).

To date, the importance of Ras in intracellular signaling has been established, with Ras mediating diverse biological responses such as cell growth and proliferation, cell differentiation , and oocyte maturation. In addition, mutated forms of Ras have been found in numerous rodent and human tumors, thus establishing ras as a potent oncogene (Der and Cooper, 1983; Der et al., 1982). The oncogenic activity of Ras is

the result of point mutations that result in single amino acid substitutions converting normal Ras to a constitutively active form. Mutations that affect Ras protein structure and function have two biochemical effects: they either decrease the GTPase activity of the protein, or they increase the nucleotide exchange rate for free GTP. Both types of mutations result in prolonged and unregulated activation of the Ras protein leading to cell transformation (Bourne, 1991).

In summary, members of several groups of proto-oncogenes such as, receptor and nonreceptror tyrosine kinases, GTP binding proteins, serine/threonine kinases, and nuclear transcriptional factors, function in the transduction of signals through multiple pathways that regulate proliferation of normal cells . In spite of the fact that many intermediate regulatory steps remain to be determined, there is a clear link between activation of cell surface molecules and transient induction of nuclear regulatory factors. These nuclear factors then represent terminal elements in signaling pathways that activate critical target genes, converting the transient action of plasma membrane-originating signals to long term alteration in gene expression and other cellular responses.

In the present study, techniques in molecular biology were utilized to examine involvement of known intracellular intermediates in signaling pathways initiated by the nonreceptor protein tyrosine kinase v-Fps, whose properties will be described in subsequent portions of the manuscript. The general approach involved induction of gene expression through activation of the tyrosine kinase activity of a ts derivative of v-Fps. Inhibitors specific for putative intermediates that interfere with induction of transcription were used, to implicate these molecules in v-Fps-initiated signal transduction.

PART ONE: EVIDENCE THAT A G-PROTEIN TRANSDUCES SIGNALS INITIATED BY THE PROTEIN-TYROSINE KINASE V-FPS*

INTRODUCTION

Fujinami Sarcoma Virus (FSV) encodes a phosphoprotein, v-Fps, with a kinase activity that phosphorylates proteins on tyrosine residues (Feldman et al., 1980). The protein-tyrosine kinase activity of v-Fps can cause rapid transformation both in vivo and in vitro (Hanafusa et al., 1980). PTK activity is associated with early events in intracellular signaling in response to extracellular stimuli (Drucker et al., 1989). In an effort to elucidate the mechanisms through which activation of PTK activity of v-Fps leads to transformation, we have utilized pharmacological and physiological inhibitors of intracellular intermediates known to play a role in cellular proliferation, and assessed their effect on the induction of early response genes by v-Fps. Thus, we previously demonstrated (Spangler et al., 1989) that the oncogenic PTK v-Fps uses a PKC-mediated signaling pathway to induce the expression of a reporter gene, 9E3, whose expression correlates with transformation (Sugano et al., 1987; Bedard et al., 1987). PKC is a serine-threonine kinase that is activated in response to phospholipid breakdown mediated by phospholipases and GTP-binding proteins coupled to cellular surface receptors.

G-proteins are a class of regulatory molecules activated by the exchange of GDP for GTP, and inactivated by the hydrolysis of GTP to GDP (Gilman, 1987; Bourne et al., 1990). There are two major classes of G-proteins: the heterotrimeric class, frequently involved in signals mediated by receptors containing seven transmembrane domains; and the monomeric class, represented by the Ras gene family and elongation

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factor-Tu (Gilman, 1987; Bourne et al., 1990). The involvement of G-proteins of the heterotrimeric class in the transduction and amplification of intracellular signals has largely been restricted to signals initiated by membrane receptors of the seven transmembrane domain class (Bourne et al., 1991). However, there have been reports suggesting the involvement of heterotrimeric class G-proteins in some of the signals activated by the insulin receptor, the epidermal growth factor receptor, and the fms oncogene product, which have PTK activity (Johnson et al., 1986; Gawler et al., 1987; Krupinski et al., 1988; Luttrell et al., 1090; Imamura and Kufe, 1988; Imamura et al., 1990). Although the involvement of G-proteins in PTK-induced signals is not clear, G-proteins have been implicated in the activation of PKC (Lapetina and Reep, 1987; Fain et al., 1988; Erusalimsky et al., 1988). We therefore sought to determine whether a G-protein is required for v-Fps to induce PKC activity and gene expression.

RESULTS

GTPγS activates 9E3 gene expression. Non-hydrolyzable analogues of GTP, such as GTPγS, have been used to demonstrate the involvement of G-proteins in intracellular signaling pathways (Gilman, 1987; Gomperts, 1983; Burch and Axelrod, 1987; Erusalimsky et al., 1988). The inability to hydrolyse GTP maintains G-proteins in the activated state (Gilman, 1987). The addition of GTPγS to permeabilized CEF induced expression of the transformation-related 9E3 gene while having no effect upon the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 1). These data suggested the involvement of a G-protein in the signaling pathway(s) that lead(s) to expression of 9E3. We previously demonstrated that the PTK v-Fps induces 9E3 gene expression via a PKC-mediated intracellular signaling pathway (Spangler et al., 1989). PKC is activated by diacylglycerol generated by phospholipid metabolism (Nishizuka, 1986). Since G-proteins have been implicated in the induction of

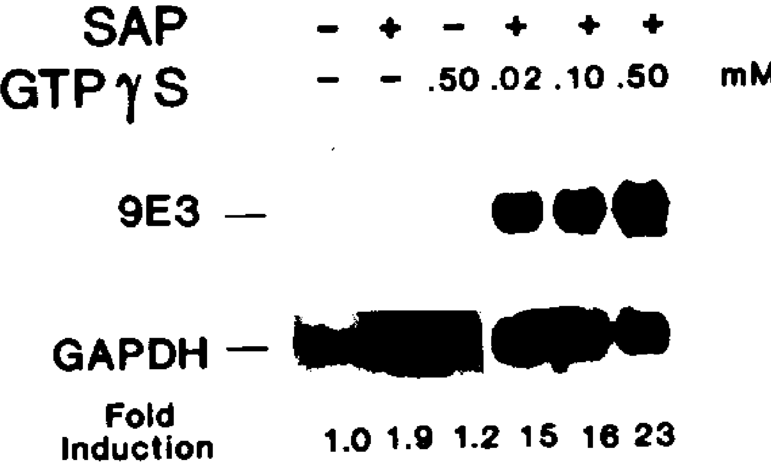


Figure 1. Induction of 9E3 gene expression by GTP S. Confluent CEF were permeabilized in the presence of increasing concentrations of GTPγS (uM). After permeabilization, cells were rinsed twice with serum-free media containing GTPγS as indicated, and the cells were incubated for another 90 min. Total RNA was extracted and analyzed by Northern blot analysis using radioactively labeled 9E3 DNA as a probe. To normalize for the RNA content, the filter was washed as previously described (Qureshi et al., 1991) and reprobed with radioactively labeled GAPDH DNA (Fort et al., 1985). Numbers at the bottom of the figure reflect the relative band intensities of 9E3 RNA relative to GAPDH RNA as determined by densitometer tracing. The fold increase values were normalized relative to the control lane which was assigned a value of 1.

phospholipid metabolism (Lapetina and Reep, 1987; Fain et al., 1988; Hurst et al., 1990), we investigated whether a G-protein functions as an intermediate in v-Fps-induced PKC activation and 9E3 expression.

GDPßS inhibits v-Fps-, but not PBt_2-induced 9E3 gene expression. To determine if v-Fps-induced expression of 9E3 requires a G-protein, we employed GDPßS, a non-hydrolyzable analogue of GDP which has been used to block signals mediated by G-proteins (Gilman, 1987; Gomperts, 1983; Burch and Axelrod, 1987; Erusalimsky et al., 1988). 9E3 gene expression was induced by v-Fps using tsNY225-infected CEF which express a temperature-sensitive derivative of v-Fps (Hanafusa et al., 1981; Spangler et al., 1989). v-Fps-induced expression of 9E3 was inhibited by GDPßS in a dose-dependent manner (Fig. 2a). GDPßS had no effect on expression of the control gene GAPDH. These data suggest that the induction of 9E3 by v-Fps requires a G-protein.

The induction of 9E3 gene expression by v-Fps requires PKC (Spangler et al., 1989). If a G-protein is functioning downstream from PKC in a v-Fps-induced signaling pathway leading to the induction of 9E3, then induction of 9E3 by direct activation of PKC should also be sensitive to GDPßS. Alternatively, if a G-protein is functioning either upstream from PKC or independently of PKC, the induction of 9E3 by direct activation of PKC should be insensitive to GDPßS. As shown in Fig. 2b, the induction of 9E3 by the PKC agonist phorbol dibutyrate (PBt_2) was not inhibited by GDPßS at concentrations that effectively inhibited v-Fps-induced 9E3. Therefore, the putative G-protein is either functioning upstream from PKC or acting independently of PKC.

GDPßS inhibits v-Fps-, but not PBt_2-induced phosphorylation of the 67-kDa PKC substrate. v-Fps induces the rapid phosphorylation of a 67-kDa PKC substrate in CEF (Spangler et al., 1989). This protein is the avian equivalent of the 80-kDa PKC substrate known as MARCKS (Stumpo et al., 1989) in mammalian cells

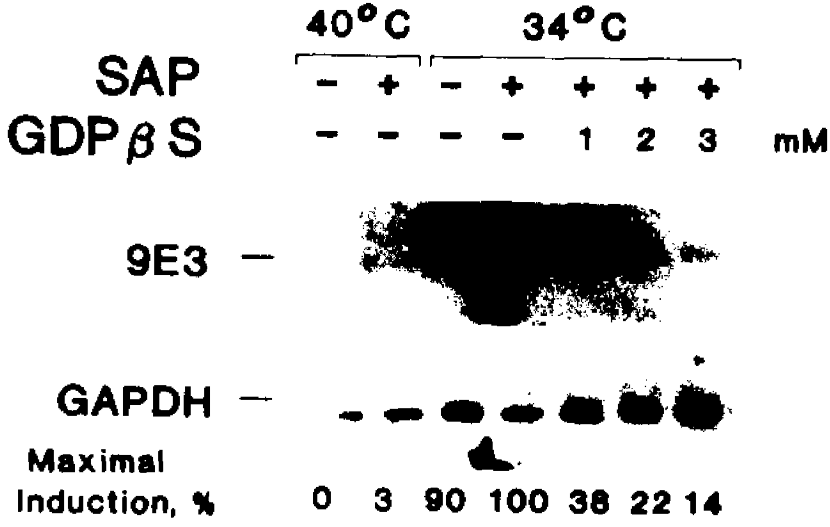


Figure 2. The effect of GDPßS on v-Fps- and PBt2-induced 9E3 gene expression in permeabilized CEF. a. tsNY225-infected CEF were permeabilized as in Fig. 1 in the presence of increasing concentrations of GDPßS (mM). Cells were then maintained at the non-permissive temperature for v-Fps (40°C) for 30 min, shifted to the permissive temperature (34°C) and incubated for another 60 min. RNA analysis was performed as in Fig. 1. RNA content was normalized as in Fig. 1 by reprobing with radioactively-labeled GAPDH DNA.

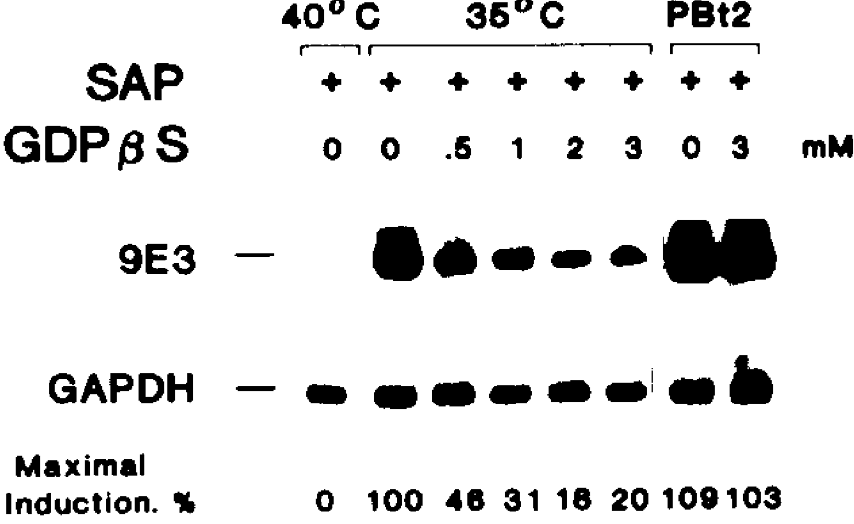


Figure 2. b. tsNY225-infected CEF maintained at 41oC were permeabilized as described above. 9E3 gene expression was then induced either by shifting the cells to 34oC or by treatment with 100 ng/ml PBt2 at 40oC. GDPßS was present at the indicated concentrations (mM). RNA was extracted for Northern analysis 60 min later. Quantitation of 9E3 RNA was relative to GAPDH RNA. Values were normalized to the lane maximally-induced by v-Fps which was assigned a value of 100%; control lanes were assigned a values of 0%. The maximum fold induction by v-Fps in a was 26; in b was 32.

(Sagara et al., 1986; Spangler et al., 1989). Both v-Fps and PBt_2 were able to induce phosphorylation of the 67-kDa PKC substrate in permeabilized cells (Fig. 3a). v-Fps-induced phosphorylation of the 67-kDa PKC substrate was inhibited by GDPßS, whereas PBt_2-induced phosphorylation of the 67-kDa PKC substrate was insensitive to GDPßS (Fig. 3b). These data suggest that the G-protein required by v-Fps for induction of 9E3 functions upstream from PKC. Consistent with the hypothesis that the G-protein functions upstream from PKC, GTPγS induced phosphorylation of the 67-kDa substrate in permeabilized CEF (Fig. 3c).

v-Fps induces increased GTP-binding to a 50-kDa protein. If v-Fps is activating a G-protein, increased binding of GTP to a G-protein should take place (Gilman, 1987; Bourne et al., 1990). tsNY225-infected CEF, maintained at either the permissive or non-permissive temperature for v-Fps, were permeabilized in medium containing a GTP photo-affinity reagent. The cells were irradiated to covalently crosslink bound GTP, and protein extracts were resolved by gel electrophoresis as above. A prominently-labeled protein migrated with an apparent molecular weight of 50-kDa. As shown in Fig. 4, increased binding of GTP to this 50-kDa protein was observed in cells with temperature-activated v-Fps PTK activity. Uninfected CEF did not show increased GTP binding to this protein upon temperature shift (Fig. 4). Increased GTP-binding to other weakly-labeled proteins was not detected under conditions used here. The apparent molecular weight of this protein is similar to the molecular weight of α-subunits of the heterotrimeric class G-proteins (Gilman, 1987).

The implicated G-protein is not a substrate for either cholera or pertussis toxins. Several heterotrimeric class G-proteins are substrates for ADP-ribosylation by bacterial toxins (Gilman, 1987). ADP-ribosylation by cholera toxin blocks GTP hydrolysis and therefore stimulates signals mediated by G-proteins that are substrates for cholera toxin. We previously demonstrated that cholera toxin does not induce phosphorylation of MARCKS protein in either avian or murine fibroblasts (Spangler

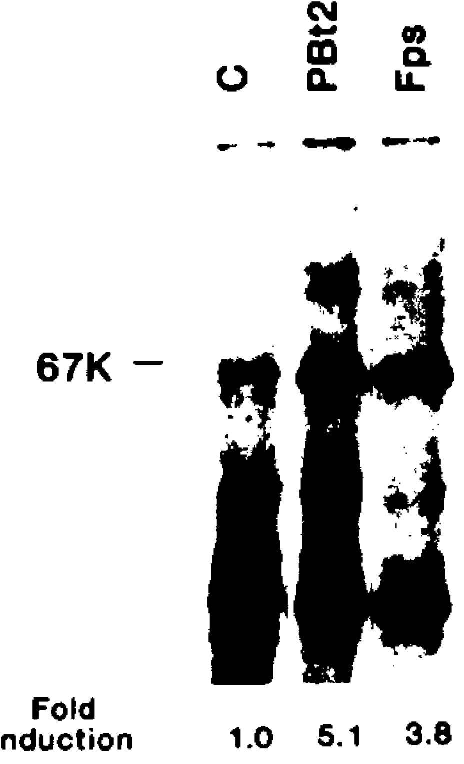


Figure 3. The effect of GDPßS on v-Fps- and PBt2-induced phosphorylation of the 67K PKC substrate. a. Induction of phosphorylation of the 67K PKC substrate by v-Fps and PBt_2 in permeabilized cells. For v-Fps-induced 67K phosphorylation, tsNY225-infected CEF were shifted from the non-permissive to the permissive temperature for 15 min prior treatment with permeabilization medium. Where indicated, PBt_2 (100 ng/ml) was added just prior to addition of permeabilization medium. After incubation for 3 min in permeabilization medium containing [γ-^{32}P]ATP, the cells were lysed and samples were analyzed by SDS-polyacrylamide gel electrophoresis.

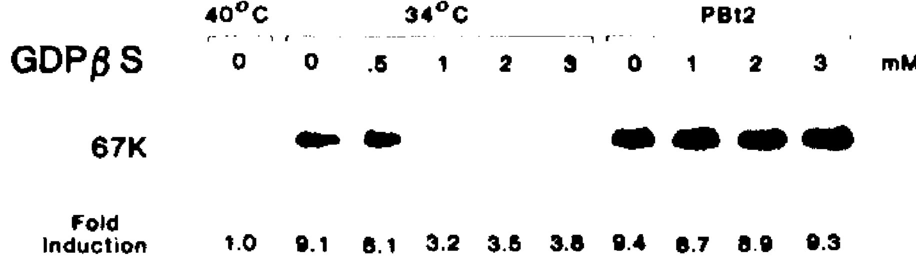


Figure 3. b. The effect of GDPßS on v-Fps and PBt_2-induced phosphorylation of the 67K PKC substrate. Induction of 67K phosphorylation was performed as in a, but in the presence of the indicated concentrations of GDPßS (mM) in the permeabilization medium.

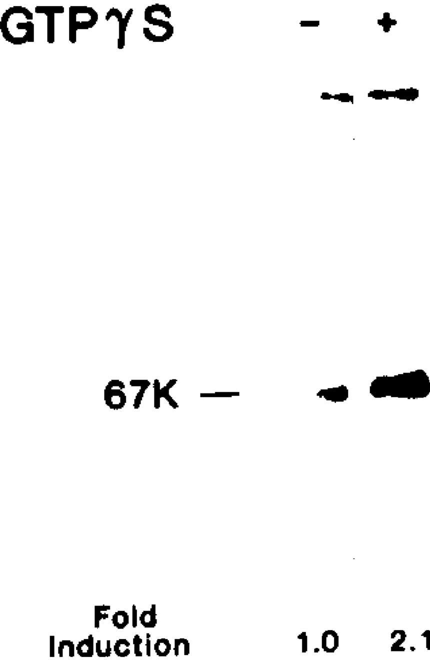


Figure 3. c. GTPγS-induced phosphorylation of the 67K PKC substrate. CEF were permeabilized in the presence and absence of GTPγS (100 uM) and phosphorylation of the 67K PKC substrate was examined as above. Numbers at the bottom reflect relative 67-kDa band intensity as determined by densitometer tracing. Values were normalized to the control lane which was assigned a value of 1. The data presented is a representative experiment that was repeated 3 times with a mean induction of 2.06 (+/- 0.13).

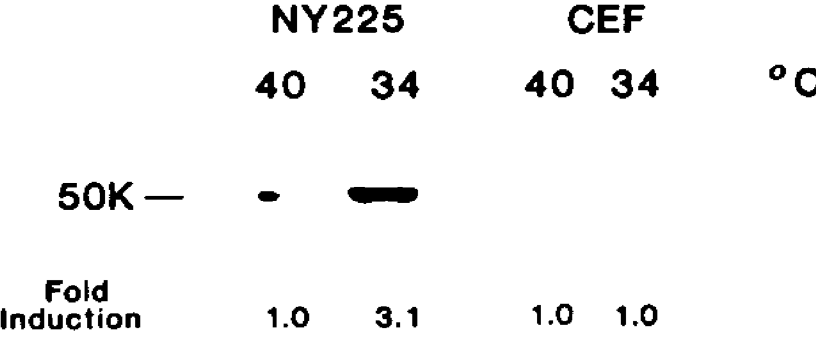


Figure 4. v-Fps-induced GTP binding to a 50K protein. CEF or tsNY225-infected CEF were shifted from the non-permissive to the permissive temperature for v-Fps for 15 min and then permeabilized for 3 min in medium containing 8-azido-[a-^{32}P]GTP. Cells were then UV-crosslinked, lysed, and subjected to electrophoresis as in Fig. 3. Quantitation of the 50-kDa band was as in Fig. 3 with the 40°C lanes being assigned values of one. The data presented is a representative experiment that was repeated 3 times with a mean induction of 2.74 (+/-0.31) upon temperature shift of tsNY225-infected CEF; for CEF, the value was 0.99 (+/-0.15) over 3 independent experiments.

et al., 1989; Qureshi et al., 1991). Under conditions where cholera toxin induced cAMP production, 9E3 gene expression was not detected (data not shown). Pertussis toxin, which blocks some G-protein-mediated signals, had no effect on v-Fps-induced expression of 9E3 and actually stimulated 9E3 expression induced by v-Src at high concentrations (data not shown). Therefore, the G-protein implicated here is not likely a substrate for either cholera or pertussis toxin.

DISCUSSION

The data presented above have shown that the non-hydrolysable analogue GTPγS induces both phosphorylation of the 67-kDa PKC substrate and 9E3 gene expression in permeabilized CEF. Both v-Fps-induced 67-kDa phosphorylation and v-Fps-induced 9E3 gene expression were sensitive to GDPβS, whereas PBt_2-induced 67-kDa phosphorylation and 9E3 gene expression were insensitive to GDPβS. These data suggest that a G-protein functions upstream from PKC in a v-Fps-initiated intracellular signaling pathway leading to the induction of 9E3 gene expression. Consistent with this hypothesis, we found increased GTP binding to a 50-kDa protein upon activating the PTK activity of v-Fps.

The increased GTP-binding to a protein with a molecular weight similar to those reported for α-subunits of heterotrimeric class G-proteins suggests that the putative G-protein may be of the heterotrimeric class. On the other hand, the observed increase in GTP-binding to the 50-kDa protein may be be unrelated to the effects observed using guanine nucleotides to activate and inhibit G-protein function. The potential involvement of a heterotrimeric class G-protein in v-Fps-induced signals does not preclude the involvement of a member of the monomeric Ras gene family in v-Fps signaling. In fact, it was previously demonstrated that microinjection of Ras antibodies blocked v-Fps-induced transformation (Smith et al., 1986). In addition, we have found

that Ras is required for v-Fps to induce PKC-independent gene expression in rat fibroblasts (Alexandropoulos et al., 1992a). Thus, v-Fps may be activating two different classes of G-proteins in two distinct signaling pathways.

Since the G-protein activated by v-Fps likely contributes to the activation of PKC, the G-protein may activate phospholipid metabolism which results in the production of diacylglycerol, the physiological activator of PKC. We recently determined that v-Src, which, like v-Fps, is a non-receptor class PTK, activates a phosphatidylcholine-specific phospholipase D that leads to the production of diacylglycerol (Song et al., 1991). v-Src also induces the expression of 9E3, and preliminary data suggest that v-Src-induced 9E3 is sensitive to GDPßS (K. Alexandropoulos and D. Foster, unpublished results). A G-protein was recently reported to induce phospholipase D-mediated hydrolysis of phosphatidylcholine (Hurst et al., 1990). Thus, the phosphatidylcholine-specific phospholipase D activated by v-Src could be a target for the G-protein activated by v-Fps.

PART TWO: V-FPS RESPONSIVENESS IN THE EGR-1 PROMOTER IS MEDIATED BY SERUM RESPONSE ELEMENTS

INTRODUCTION

To further characterize intracellular signaling by v-Fps, we have examined v-Fps responsiveness of the mitogen responsive Egr-1 gene, also known as zif/268 (Lau & Nathans, 1987), Krox 24 (Almendral, 1988), TIS 8 (Lim et al., 1987), CEF5 (Simmons et al., 1989), NGFI-A (Milbrandt, 1988) and 9II-6 (Jahner and Hunter, 1991). Egr-1 expression is activated in response to a variety of growth factors and has been shown to be involved in neuronal differentiation (for review, see Sukhatme, 1990). The Egr-1 gene encodes a transcription factor (Lemaire et al., 1990, Patwardhan et al., 1991) that recognizes a specific DNA target sequence found upstream of several cellular genes (Christy and Nathans, 1989; Cao et al., 1990). The sequence recognized by Egr-1 is also recognized by the tumor suppressor Wilms' tumor gene product (Rauscher et al., 1990; Madden et al., manuscript submitted). Egr-1 was recently shown to be induced by v-Fps (Jahner and Hunter, 1991). Thus, it is possible that Egr-1 controls secondary responses to v-Fps leading to the transformed phenotype. Data presented in this report characterize the mechanism through which v-Fps activates Egr-1. Thus, activation of Egr-1 by v-Fps is rapid and transient and is independent of PKC. Furthermore, induction is mediated by clustered serum response elements that contain the conserved motifs $CC(A/T)_6GG$ known as CArG boxes (C A/T rich G).

RESULTS

v-Fps transcriptionally activates Egr-1 gene expression. The induction of "primary response" genes [those induced in the absence of protein synthesis

(Herschman, 1989)] is a useful early phenotype for the study of intracellular signal transduction pathways. A primary response to all mitogenic stimuli examined thus far is the induction of the transcription factor Egr-1 (Sukhatme, 1990). We therefore examined whether Egr-1 gene expression was induced in response to v-Fps activation. In order to study v-Fps-induced intracellular signaling pathways, we used 3Y1-NY225 cells which express a temperature-sensitive v-Fps mutant whose kinase activity is rapidly activated after temperature shift (Hanafusa et al., 1981; Birnbaum et al., 1987). 3Y1-NY225 cells maintained at the non-permissive temperature for v-Fps ($40^{o}C$) were shifted to the permissive temperature ($35^{o}C$) and levels of Egr-1 RNA were determined using "northern-blot" analysis. Activating the kinase activity of v-Fps resulted in a transient increase in Egr-1 RNA levels that peaked at 30 min (Figure 5a). v-Fps induced Egr-1 gene expression was independent of protein synthesis since pretreatment of cell cultures with cycloheximide, which inhibits protein synthesis, did not interfere with v-Fps-induced Egr-1 expression (Figure 5b). The parental 3Y1 cells did not show increased levels of Egr-1 mRNA upon temperature shift (Figure 5b). Thus, a primary response to increased v-Fps kinase activity is the induction of the mitogen-responsive transcription factor Egr-1.

To determine whether v-Fps-induced Egr-1 was at the level of transcription, we subjected 3Y1-NY225 cells to nuclear "run-on" analysis (Greenberg and Ziff, 1984). Radioactively labeled RNA from nuclei isolated from 3Y1-NY225 cells maintained at both non-permissive and permissive temperatures for v-Fps was hybridized to Egr-1 and control DNAs. Activating v-Fps led to an increase in Egr-1 transcription that was similar to the increase in Egr-1 RNA levels in response to v-Fps (Figure 6). Thus, the increase in Egr-1 transcripts induced by v-Fps can largely be explained at the level of transcription.

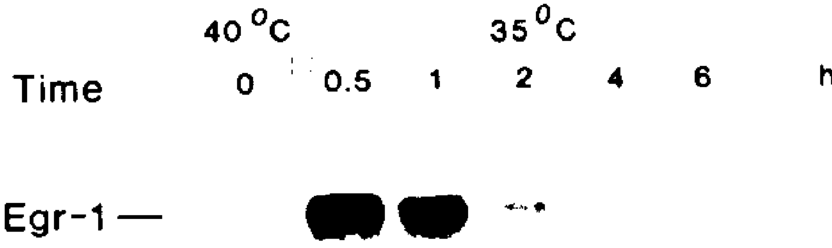


Figure 5. v-Fps induces increased levels of Egr-1 RNA. a. Quiescent NY225-3Y1 cells were shifted from the non-permissive (40^oC) to the permissive temperature (35^oC) for v-Fps for the indicated times (min). RNA was harvested, electrophoresed and hybridized to radiolabeled Egr-1 DNA as described in Materials and methods.

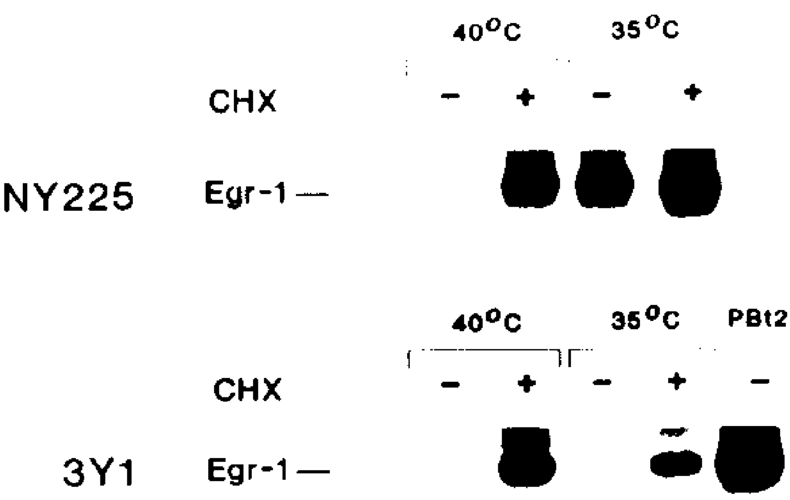


Figure 5. b. Quiescent NY225-3Y1 cells were shifted from the non-permissive to the permissive temperature for v-Fps for 30 min in the absence or presence of cycloheximide (CHX) (10 ug/ml) and Egr-1 RNA levels were determined as in (a). Egr-1 RNA levels were also examined in parental 3Y1 cells under the same conditions. Phorbol dibutyrate (PBt2) (0.1 ug/ml) was used to induce Egr-1 RNA (Lim et al., 1987) in 3Y1 cells as a positive control.

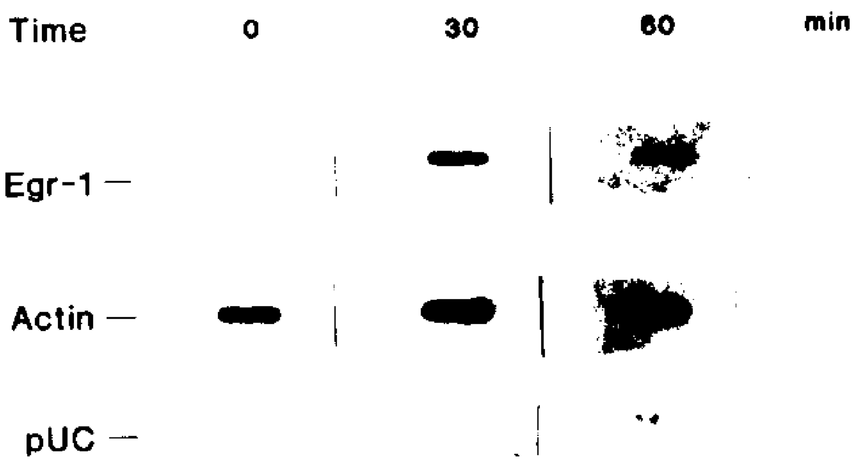


Figure 6. v-Fps-induces Egr-1 expression at the transcriptional level. "Run-on" transcription in isolated nuclei was examined in 3Y1-NY225 cells at the times indicated (min) after shifting from the non-permissive to the permissive temperature. Transcription of Egr-1 is compared with transcription of beta actin. pUC-18 vector DNA was used as a negative control.

v-Fps-induced Egr-1 expression is independent of PKC. Egr-1 has been shown to be induced by phorbol esters that activate PKC (Lim et al., 1987; Sukhatme et al., 1987). We previously demonstrated that v-Fps uses a PKC mediated signaling pathway to induce gene expression in chicken fibroblasts (Spangler et al., 1989). We therefore wished to determine whether v-Fps-induced Egr-1 was mediated by PKC. To examine whether PKC was required for v-Fps to induce Egr-1 expression, 3Y1-NY225 cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) for prolonged periods of time to deplete cells of PKC (Rodriguez-Pena and Rozengurt, 1984). Depleting cells of PKC had no effect on v-Fps-induced Egr-1 expression, whereas the induction of Egr-1 by phorbol dibutyrate was completely abolished (Figure 7). Thus, v-Fps-induces Egr-1 expression via an intracellular signaling pathway that is independent of PKC.

Activation of the Egr-1 promoter by v-Fps. To characterize the mechanism through which v-Fps activates Egr-1, we looked at the responsiveness of Egr-1 promoter sequences to v-Fps. pEgr-1 P1.2 contains Egr-1 promoter sequences between -957 and +248 relative to the transcriptional start site (Gius et al., 1990), cloned into pCAT3M (Laimins et al., 1984) upstream from the CAT gene (Figure 8a). pEgr-1 P1.2 was cotransfected into NIH 3T3 cells with pIV2.3 (Sadowski et al., 1986), which expresses v-Fps under the control of the SV40 promoter (Figure 8a). 48 hours post-transfection, cell extracts were assayed for CAT activity. As shown in Figure 8b, expression of the CAT gene from pEgr-1 P1.2 was dramatically increased in the presence of v-Fps. Cotransfection of pEgr-1 P1.2 with the parental vector (pSV_2neo) had no significant effect on CAT gene expression (Figure 8b). Thus, v-Fps is able to increase transcription from the Egr-1 promoter.

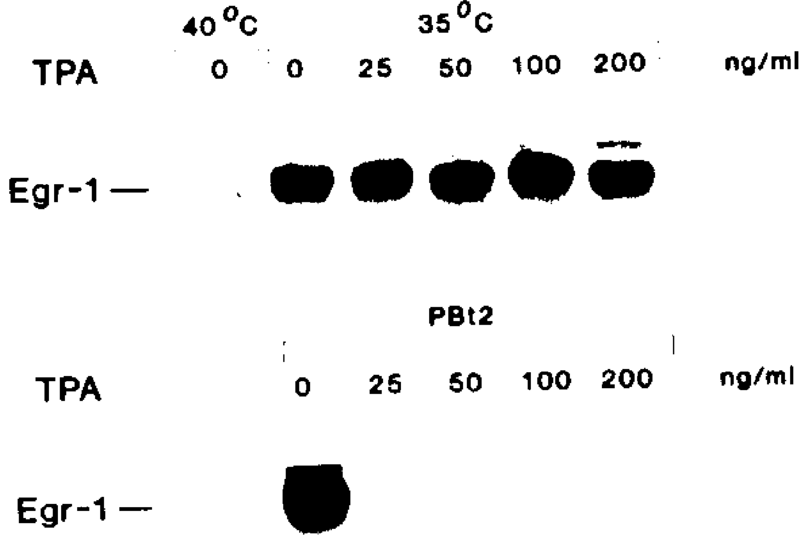


Figure 7. v-Fps-induced Egr-1 expression is independent of PKC. Cells were treated with increasing concentrations of TPA (ng/ml) to deplete cells of PKC as described previously (Qureshi et al., 1991b). TPA was added to 3Y1-NY225 cell cultures for 26 h prior to either temperature shift or addition of phorbol dibutyrate. Egr-1 RNA levels were measured as in Figure 5 after 30 min induction by either temperature shift or phorbol dibutyrate (PBt2) (0.1 μg/ml) at 40°C.

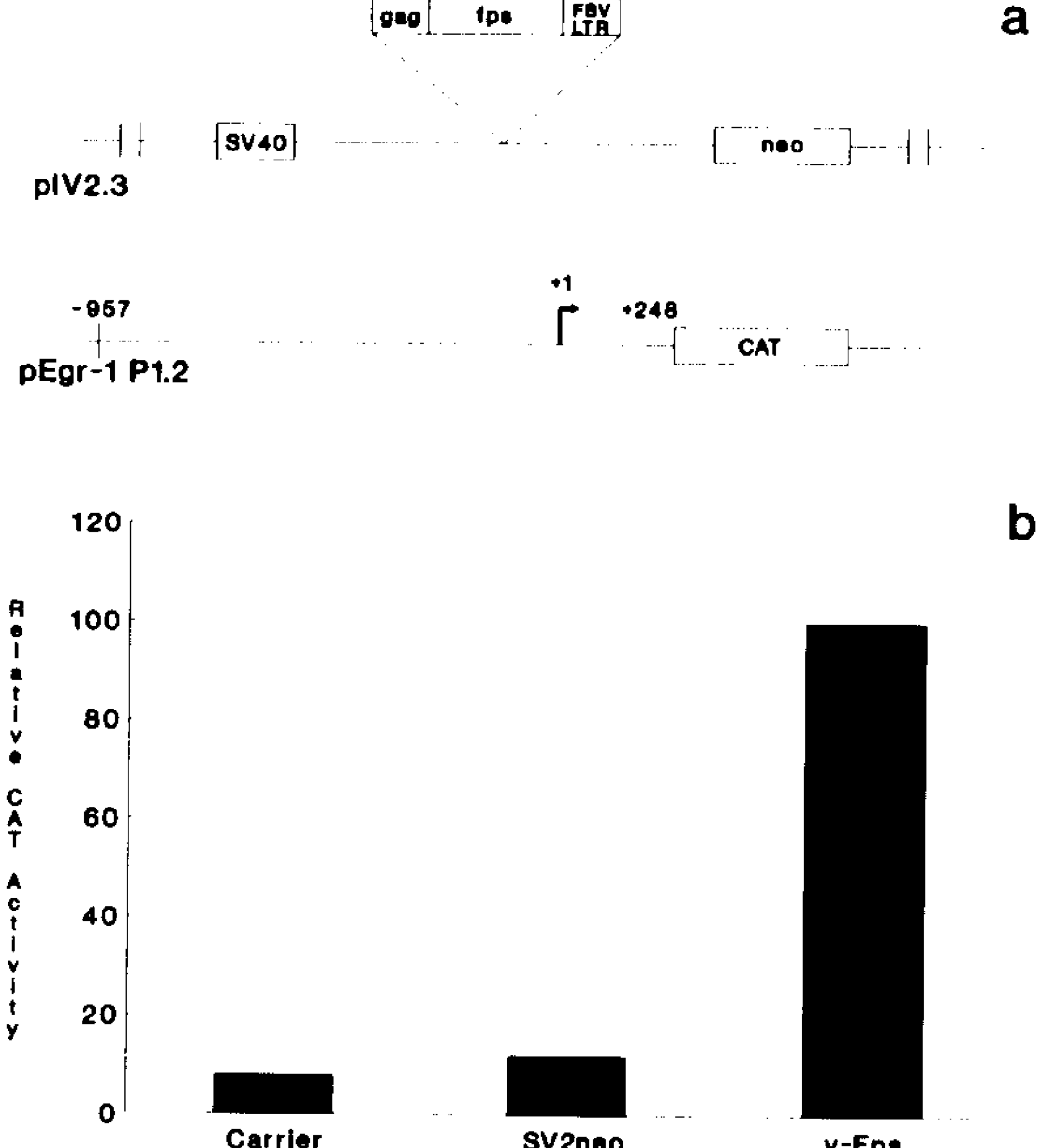


Figure 8. Egr-1 promoter activation by v-Fps. pEgr-1 P1.2 contains the CAT gene under control of the Egr-1 promoter (Gius et al., 1990). CAT activity was assayed in extracts from NIH 3T3 cells that had been transfected with pEgr-1 P1.2 and either pIV2.3, a v-Fps expression vector (Sadowski et al., 1986), pSV2neo, the vector used to construct pIV2.3, or salmon sperm carrier DNA. Transfections were performed using the calcium phosphate co-precipitation technique as described in Materials and methods. 20 h later the cells were washed and CAT activity was assayed 24 h after washing using the fluor diffusion assay (Newman et al., 1987). a. Diagram of constructs used. Bent arrows represent translational start sites. b) Relative CAT activity. Values were normalized to the CAT activity generated in the presence of pIV2.3 (v-Fps) which was assigned a value of 100. Data are the mean of at least four experiments. (S.D. carrier +/-2; SV2neo +/-6).

v-Fps-responsiveness resides in a region containing multiple SREs. To investigate the mechanism through which v-Fps activates the Egr-1 promoter, we used several constructs containing deletions in the Egr-1 promoter of pEgr-1 P1.2 (Figure 9a). p△Egr-1 P1.2, which lacks all the SREs with their core CArG boxes, but retains AP-1 and SP-1 sites (Gius et al., 1990, Qureshi et al., 1991), was cotransfected with pIV2.3 (v-Fps). As shown in Figure 9b, deletion of this region containing 6 CArG boxes reduced CAT activity to basal levels. Deletion of the AP-1 and Sp-1 sites retained in p△Egr P1.2 had no effect on CAT expression (see construct pE425, Figure 9a and 9b). Sequential deletion of the SREs from the 5'end of pE425 (Gius et al., 1990) resulted in progressive loss of CAT activity. Deletion of two SREs immediately upstream of the Egr-1 TATA box, had no effect on the levels of activation. These data show that the Egr-1 upstream sequences contain a v-Fps responsive region which is localized between nucleotides -425 and -250. This region contains four tandemly repeated SREs.

To determine if the four SREs contained within this region of the Egr-1 promoter were sufficient to confer v-Fps responsiveness, we cloned different segments of this region onto the heterologous herpes thymidine kinase minimal promoter as described in Materials and Methods. pE425/250TKCAT (Figure 10a) contains the four distal SREs whose deletion diminished v-Fps-responsiveness of pEgr-1 P1.2. Activation of CAT gene expression by v-Fps from pE425/250TKCAT was comparable to that observed for pEgr-1 P1.2 and pE425 (Figure 10b). Similarly, sequential removal of the distal SREs from the PTK35CAT constructs diminished v-Fps responsiveness (Figure 10b). Thus, the SREs localized between -425 and -250 are not only necessary for induction of Egr-1 by v-Fps, but also sufficient.

v-Fps activates gene expression controlled by individual SREs contained within the v-Fps-responsive region of the Egr-1 promoter. The data presented in Figure 10, suggest that v-Fps-induced Egr-1 expression is mediated by SREs.

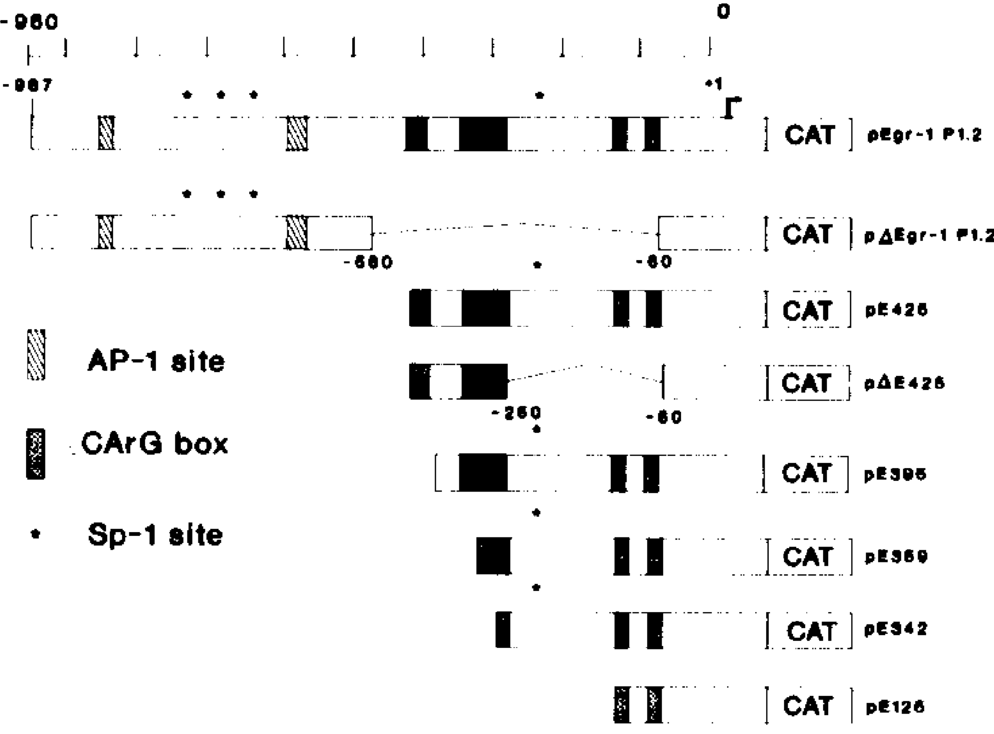


Figure 9. Deletion mutant analysis to the Egr-1 promoter reveals a v-Fps-responsive region. NIH3T3 cells were transfected with pEgr-1 P1.2 and derivatives containing various Egr-1 promoter deletions (Gius et al., 1990) along with pIV2.3. a. CAT expression vectors used. The location of cis transcriptional control elements in the Egr-1 promoter are shown in the diagram of the parental pEgr-1 P1.2 plasmid from which the deletion mutants were constructed.

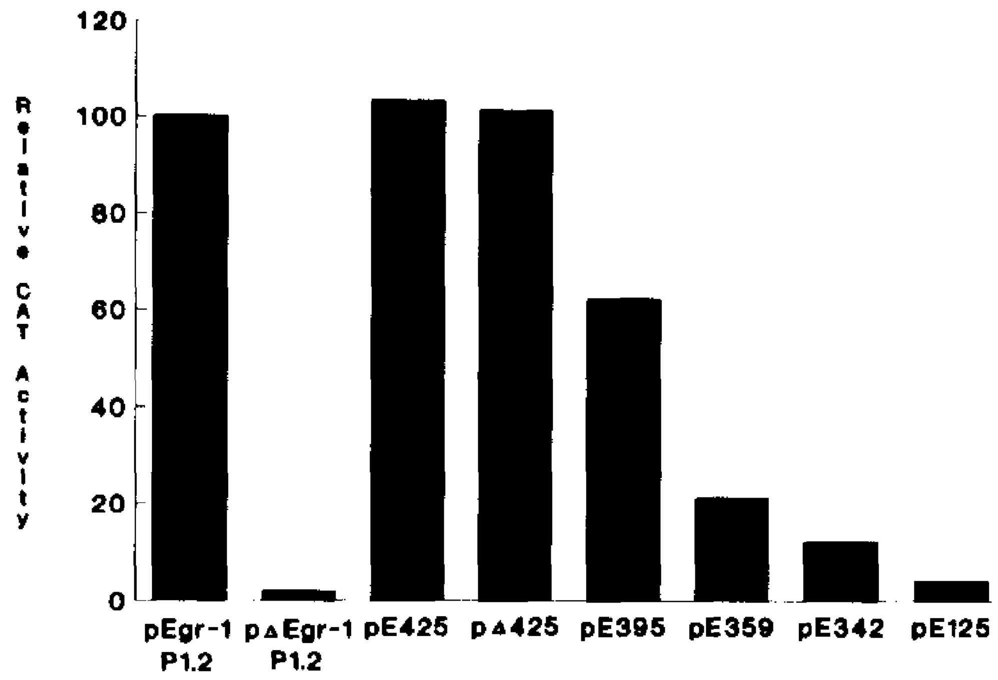


Figure 9. b. CAT activity was determined and normalized to the amount of CAT activity generated in the presence of pIV2.3 and pEgr-1 P1.2 as in Figure 4. Basal CAT activity generated by pEgr-1 P1.2 in the absence of v-Fps was equivalent the CAT activity observed for p Egr-1 P1.2. Data are the mean of at least four independent experiments. (S.D. ΔEgr-1 +/-1; 425+/-15; Δ425 +/-22; 395 +/-6; 359 +/-9; 342 +/-7; 125 +/-4).

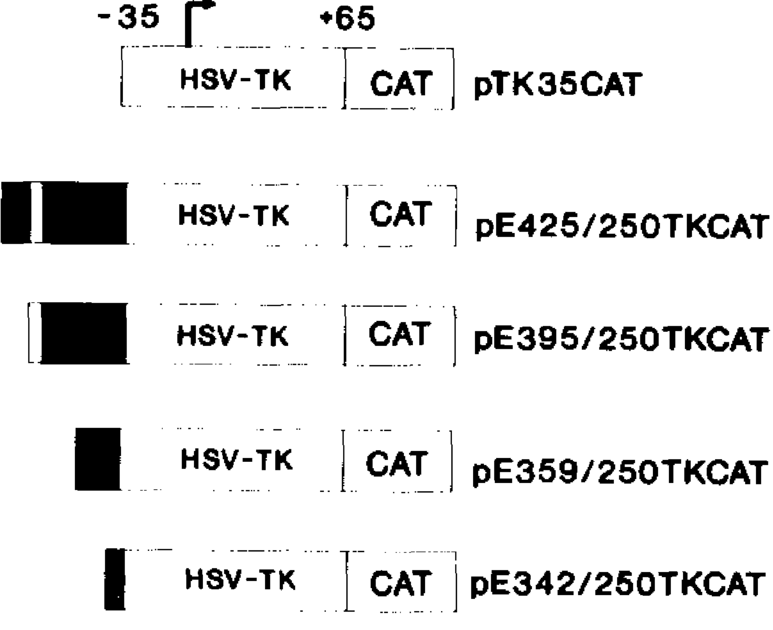


Figure 10. The four SREs contained between -425 and -250 are sufficient for conferring v-Fps-responsiveness. NIH 3T3 cells were transfected with pIV2.3 and constructs containing the deletions of pE425, pE395 and pE359 fused to the HSV-TK35CAT plasmid, and CAT activity was assayed as in Fig. 8b. a. CAT expression vectors used.

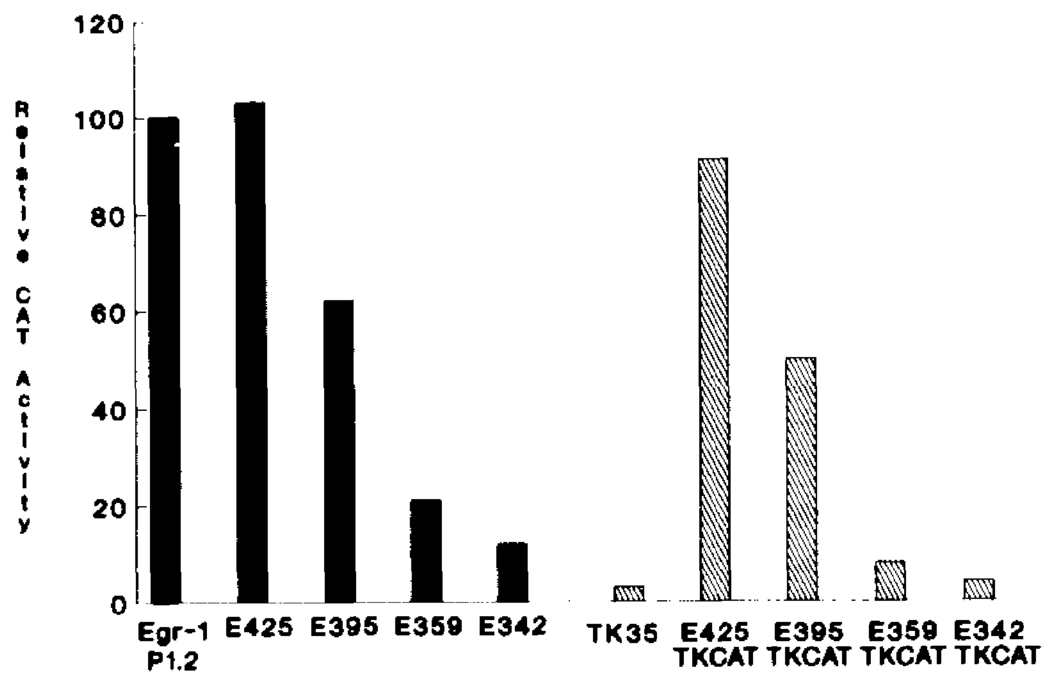


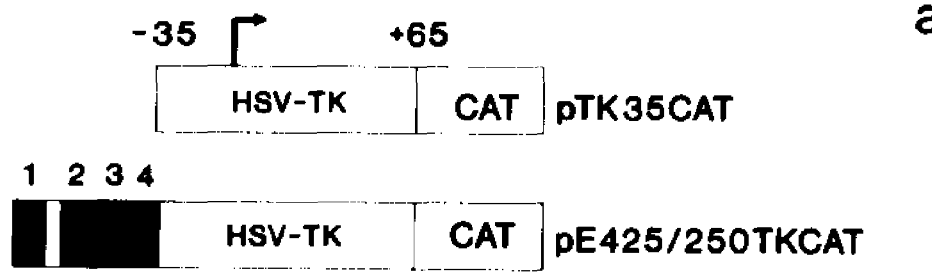
Figure 10. b. CAT activity from cells transfected with pEgr-1 P1.2 deletion constructs and TK35CAT constructs was assayed and normalized to the amount of CAT activity generated in the presence of pIV2.3 and pEgr-1 P1.2. Basal activity generated by the TK35CAT constructs in the absence of v-Fps was equivalent to that observed for TK35CAT. Data are the mean of at least four independent experiments. (S.D. solid bars: 425 +/-15; 395 +/-6; 359 +/-9; 342 +/-7; hatched bars: TK35 +/-1; 425 +/-18; 395 +/-15; 359 +/-2; 342 +/-2).

We therefore, examined whether individual SREs from this region could respond to v-Fps. Several constructs were made containing individual SREs upstream from the HSV-TK promoter (Figure 11a). pTKCATSRE1 contains the most distal SRE from the v-Fps-responsive region. This SRE was able to confer v-Fps-responsiveness to the HSV-TK minimal promoter (Figure 11b). However, the level of conferred responsiveness was substantially lower than that conferred by the collection of four SREs that gave maximal v-Fps-responsiveness (Figure 11b). pTKCATSRE2 contained the second most distal SRE upstream from the HSV-TK promoter was also weakly responsive to v-Fps. pTKCATSRE1-2 which contains the two most distal SREs with the short intervening sequence, was more responsive than either of the two SREs by themselves. pTKCATSRE3 containing the third SRE, was also weakly responsive to v-Fps. pTKCATSRE4 gave little or no response to v-Fps. Thus, the individual SREs in the v-Fps-responsive region were not as responsive to v-Fps as the collection of SREs in this region were.

The data presented above suggest that activating the PTK activity of v-Fps results in the activation of early response genes via two different pathways: 1)a PKC-dependent pathway mediated by a G-protein which activates PKC, and 2) a PKC-independent pathway which has not been characterized.

DISCUSSION

In this study, we have demonstrated that v-Fps induced expression of the mitogen-responsive transcription factor Egr-1 is a primary response to v-Fps that does not require protein synthesis. Deletion analysis of the Egr-1 promoter showed that v-Fps responsiveness was localized to a cluster of four CArG box-containing SREs located between -425 and -250 relative to the transcription start site. This region



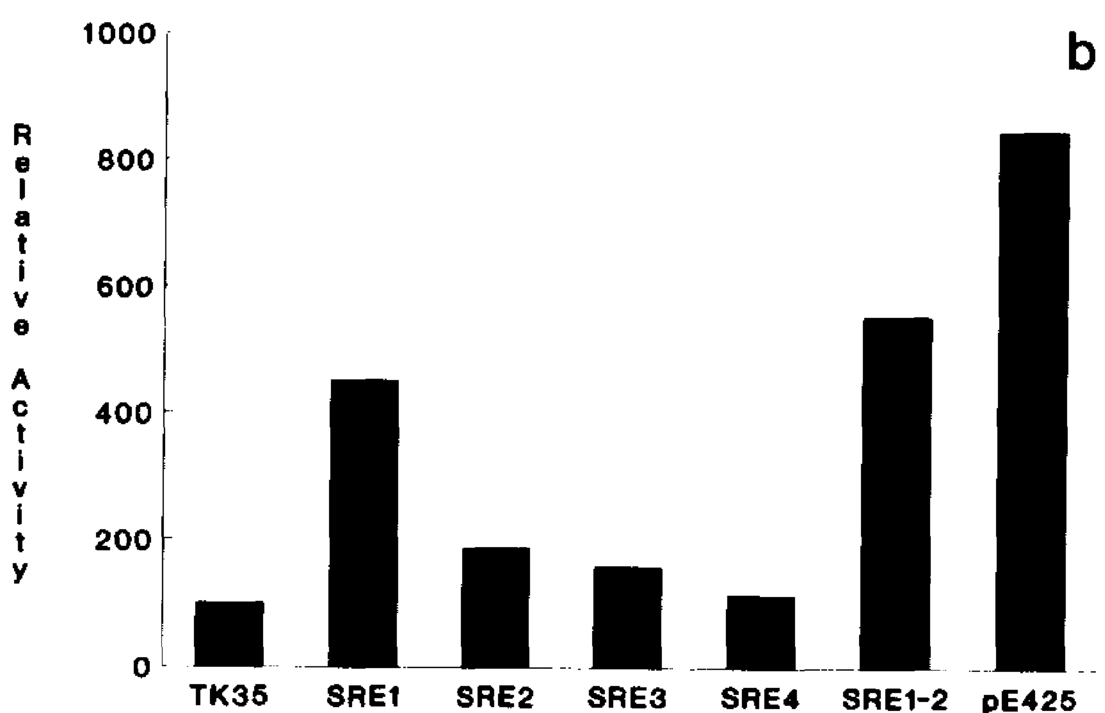


Figure 11. Individual SREs from the v-Fps-responsive region of the Egr-1 promoter confer v-Fps-responsiveness. NIH 3T3 cells were transfected with pIV2.3 and constructs containing individual SREs from the v-Fps-responsive region of the Egr-1 promoter fused to the pTK35CAT plasmid. a. Diagram of plasmids used to make pTKCATSRE1,2,3,and4. b. CAT activity from cells transfected with pTKCATSRE constructs containing individual SREs in the presence of pIV2.3 was assayed and normalized to the amount of CAT activity generated in the presence of pIV2.3 and pTK35CAT. Data are the mean of at least four independent experiments. (S.D. SRE1 +/- 87; SRE2 +/-40; SRE3 +/-26; SRE4 +/- 36; SRE1-2 +/-79; 425 +/-57).

was sufficient to confer v-Fps-responsiveness to a heterologous promoter. Although, v-Fps was able to activate individual SREs from the v-Fps responsive region of the Egr-1 promoter, the presence of a cluster of SREs was important for v-Fps-responsiveness. These data suggest that v-Fps transcriptionally activates Egr-1 via this cluster of SREs. We previously demonstrated that this cluster of SREs mediated responsivenes the oncogenic protein tyrosine kinase v-Src. This may reflect common signaling mechanisms by protein-tyrosine kinases for activating the Egr-1 promoter. In this regard, it is important that the Egr-1 promoter contains other transcriptional control elements including two activating protein-1 (AP-1) sites. AP-1 sites are consesus sequences that are activated by agonists of protein kinase C (Angel et al., 1987). Both v-Fps (Spangler et al., 1989) and v-Src (Spangler et al., 1989, Qureshi et al., 1991) activate protein kinase C-mediated intracellular signals. Thus, the activation of the Egr-1 promoter via SREs rather than AP-1 sites suggests a complex selectivity by the intracellular signals initiated by v-Fps and perhaps other protein-tyrosine kinases.

Serum, growth factors and phorbol esters have also been shown to induce Egr-1 SREs (Christy and Nathans, 1989). Induction was shown to be mediated by serum response factor (SRF) (Christy and Nathans, 1989), a factor that binds to CArG boxes contained within SREs (Treisman, 1986, Prywes et al., 1988, Treisman, 1990). Thus, it is possible that v-Fps-induced Egr-1 involves the activation of SRF. The fourth SRE, by itself, was not responsive to v-Fps. Consistent with an SRF requirement for the v-Fps activation of Egr-1, the CArG box of SRE4, has only 5 A/Ts between the CC and GG of the CArG box and does not bind SRF (Christy and Nathans 1989). The differential activation of the remaining SREs within the Egr-1 promoter that do bind SRF suggests that sequences flanking the CArG boxes could be important for responsiveness to v-Fps and that additional factors may be involved. The intracellular components that contribute to the activation of SRF are not known; however SRF requires phosphorylation for induction of transcription (Prywes et al., 1988); thus a

protein kinase may be involved. Other v-Fps-induced factors that might activate, or function synergistically with SRF to induce Egr-1, remain to be determined.

PART THREE: V-FPS INDUCES EGR-1 EXPRESSION VIA AN INTRACELLULAR SIGNALING PATHWAY THAT IS INDEPENDENT OF PROTEIN KINASE C AND DEPENDENT UPON HaRAS AND RAF-1

INTRODUCTION

PKC-independent pathways are poorly understood. Taking advantage of the v-Fps-induced activation of the Egr-1 promoter, we will examine the role of cellular intermediates thought to function as signal transducers involved in cell growth and transformation (Fig 12). Such intermediates include c-Ras (reviewed by Barbacid, 1987) and c-Raf-1 kinase (reviewed by Li et al., 1991; Rapp, 1991). c-Ras, a GTP-binding protein, has been shown to be required for cell proliferation (Mulcahy et al., 1985; Feig and Cooper, 1988), as well as in transformation by a variety of oncogenes such as src, fms, and fes/fps (Smith et al., 1986). v-Fps-induced transformation has be shown to be sensitive to Ras antibodies when these are microinjected into cells (Smith et al., 1986). In addition, v-Fps phosphorylates GAP, a protein that regulates the GTPase activity of Ras, on tyrosine residues (Ellis et al., 1990).

On the other hand, c-Raf-1, a serine-threonine kinase, is activated in response to a variety of growth factors such as platelet-derived growth factor (PDGF)(Morrison et al., 1989), epidermal growth factor (EGF) (Morrison et al., 1989), and colony-stimulating factor (CSF)(Baccarini et al., 1990), as well as by oncogenes such as Src (Morrison et al., 1989). In addition, c-Raf-1 was recently shown to be required for growth of normal NIH 3T3 cells when they are induced by serum or TPA, and for transformation of cells by Ki- and Ha-Ras oncogenes (Kolch et al., 1991).

In order to implicate Ras and Raf as intermediates in v-Fps signaling, we used dominant inhibitors in order to block the action of endogenous c-Ras and c-Raf-1, and

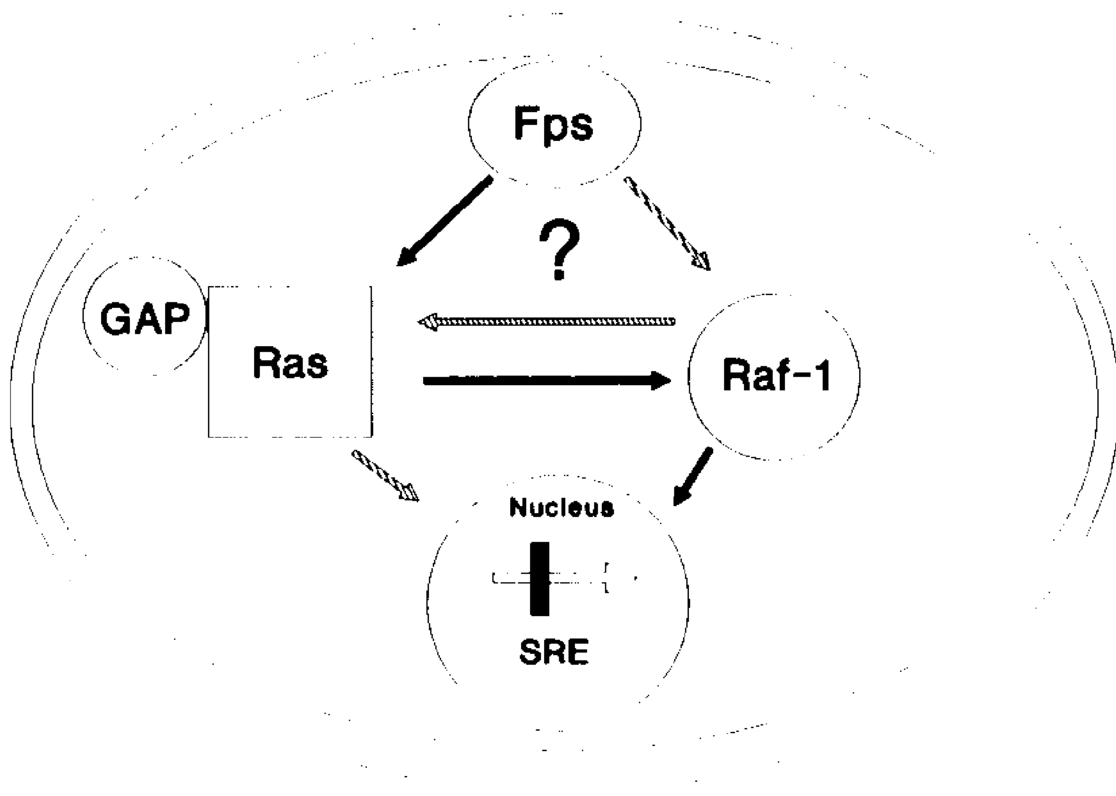


Figure 12. Model for involvement of cytoplasmic intermediates in v-Fps signaling.

consequently examined the effect of this inhibition on v-Fps-induced activation of the Egr-1 promoter.

RESULTS

Egr-1 promoter activation by v-Fps and activated forms of HaRas, and Raf-1. HaRas and Raf-1 have been implicated in protein-tyrosine kinase-induced transformation (App et a., 1991, Baccarini et al., 1990, Ellis et al., 1990, Morrison et al., 1989, Morrison et al., 1988, Mulcahy et al., 1985, Rapp, 1991, Smith et al., 1986). Dominant inhibitory mutants for HaRas (Cai et al., 1990, Feig and Cooper, 1988) and Raf-1 (Kolch et al., 1991) have been characterized, making it possible to implicate these signaling intermediates in intracellular signaling pathways. To employ the dominant inhibitory mutants for HaRas and Raf-1, we established a transient expression assay to examine the induction of Egr-1 expression. If either HaRas or Raf-1 is part of the intracellular signaling pathway activated by v-Fps leading to the induction of Egr-1, the activated oncogenic forms of HaRas and Raf-1 might also induce Egr-1 expression. pEgr-1 P1.2 (described above), is a plasmid vector that contains Egr-1 promoter sequences (Gius et al., 1990) (Fig. 12a). pEgr-1 P1.2 was cotransfected into NIH 3T3 cells with pIV2.3 (Sadowski et al., 1986), pv-HaRas (Feig and Cooper, 1988) and p3611 (Rapp et al., 1983) (Fig. 12a), which express v-Fps, v-HaRas, and v-Raf-1 respectively. 48 hours post-transfection, cell extracts were assayed for CAT activity. As shown in Fig. 12b, CAT activity was increased at least 8 fold over basal levels when vectors expressing either v-Fps, v-HaRas or v-Raf-1 were cotransfected with pEgr-1 P1.2. Cotransfection with the parental vectors for v-Fps, v-HaRas or a vector expressing v-Raf-1 deletion mutant (Jamal and Ziff, 1990) had no effect on CAT activity (Fig.12b). Consistent with data presented in Fig. 7 utilizing 3Y1 cells, activation of the Egr-1 promoter by v-Fps was insensitive to depleting cells

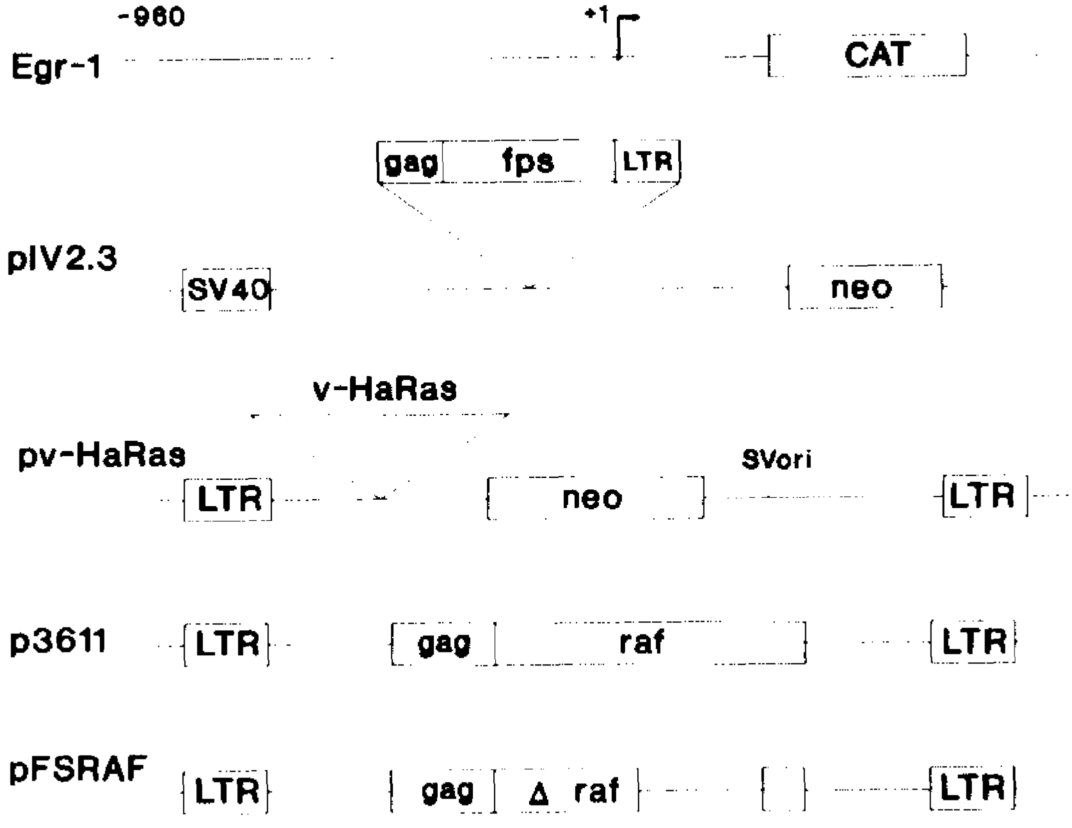


Figure 13. Activated forms of Fps, HaRas, and Raf-1 induce Egr-1 promoter activation. CAT activity was assayed in extracts from NIH 3T3 cells that had been transfected with the Egr-1 promoter-controlled CAT expression vector pEgr-1 P1.2 in the presence of vectors expressing v-Fps (pIV2.3), v-HaRas (pv-HaRas), or v-Raf (p3611). Control experiments were performed using pSV2neo and pZIPneoSV(X), the parental vectors used to construct pIV2.3 and pv-HaRas, or pFSRAF which expresses a defective deletion mutant Raf-1 protein (Jamal and Ziff, 1990). Transfections were performed as described in Materials and Methods. The transfection mixture contained 30 ug of DNA (5 ug of pEgr-1 P1.2 and 5 ug of inducer or parental vector DNA for v-Fps, v-Raf and v-Ras; carrier salmon sperm DNA was added to 30 ug). a. Diagram of constructs used. Bent arrow represents the transcriptional start site for pEgr-1 P1.2.

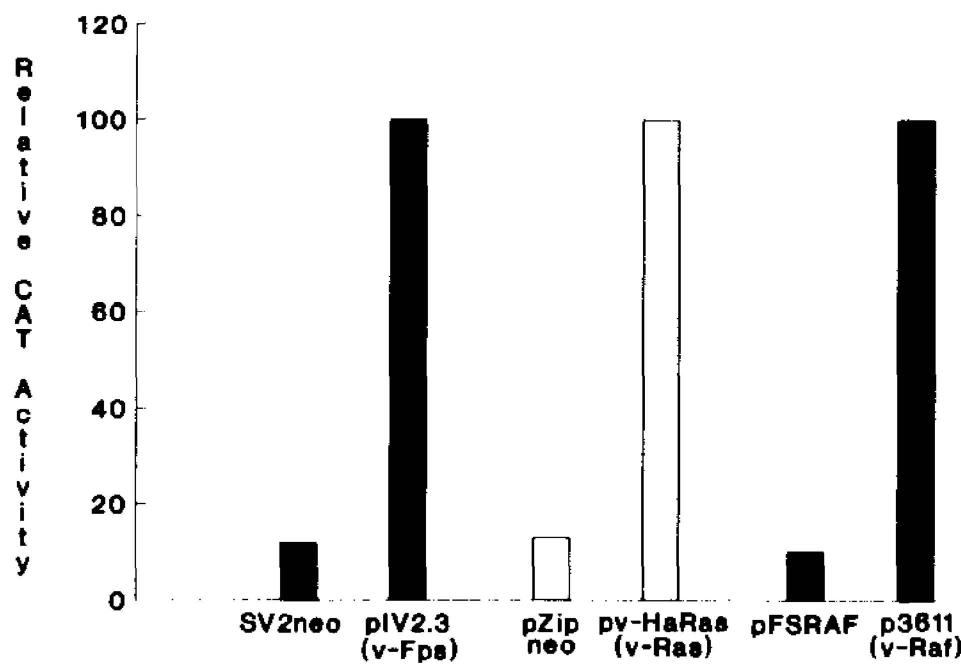


Figure 13. b. Relative CAT activity induced by the indicated DNAs. Values were normalized to the CAT activity generated in the presence of pIV2.3 (v-Fps), pv-HaRas (v-HaRas), and p3611 (v-Raf) which were assigned values of 100. Data are the mean of at least four independent experiments. (S.D. SV2neo +/-5; pZIPneo +/-3; pFSRAF +/-4).

of PKC (data not shown). The data presented in Fig. 12b show that in addition to v-Fps, activated forms of HaRas and Raf-1 induce gene expression under the control of the Egr-1 promoter in NIH 3T3 cells. Thus, it is possible that cellular forms of HaRas and Raf-1 are components of an intracellular signaling pathway activated by v-Fps leading to induction of Egr-1 gene expression.

A dominant inhibitory mutant of HaRas blocks the v-Fps but not the v-Raf activation of the Egr-1 promoter. A dominant inhibitory mutant of HaRas (Feig and Cooper, 1988) has been used to implicate cellular HaRas in intracellular signaling schemes (Cai et al., 1990). pZIP M17 expresses the mutant protein under the control of the murine leukemia virus long terminal repeat (Fig. 13a). This dominant inhibitor has a Ser for Asn substitution on residue 17 and binds GDP with high affinity. pZIP M17 was cotransfected into NIH 3T3 cells along with the v-Fps expression vector pIV2.3 and the Egr-1 promoter/CAT construct. Cotransfection of the M17 HaRas mutant reduced CAT activity induced by v-Fps to basal levels (Fig. 13b). Cotransfection of the parental plasmid used for the construction of pZIP M17, pZIP neoSV(X), had no effect on v-Fps-induced Egr-1 promoter activation (Fig. 13b). Thus, expression of the M17 HaRas mutant inhibits signals induced by v-Fps leading to the activation of the Egr-1 promoter.

We next examined the effect of the M17 inhibitory HaRas mutant on v-Raf-induced Egr-1 promoter activation. Cotransfection of the M17 HaRas mutant with p3611, which expresses v-Raf, had no effect upon v-Raf-induced Egr-1 promoter activation (Fig. 13c). This implies that the effect of the M17 HaRas mutant is not a non-specific effect upon the Egr-1 promoter and that HaRas mediates v-Fps-induced signal(s) that lead to Egr-1 promoter activation. The data also demonstrate that if Raf-1 is a component in v-Fps-induced promoter activation, it functions downstream from or independently of HaRas.

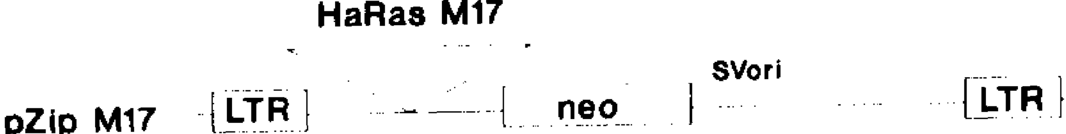


Figure 14. a. A dominant inhibitory mutant of HaRas blocks v-Fps but not v-Raf induced Egr-1 promoter activation. Transfections were performed as above, except the reaction mixture also contained either pZip M17 (5 ug), which expresses the inhibitory M17 HaRas mutant, or pZIPneoSV(X) (5 ug), the parental vector of pZip M17, as indicated. a. The structure of pZip M17 and the parental pZIPneoSV(X).

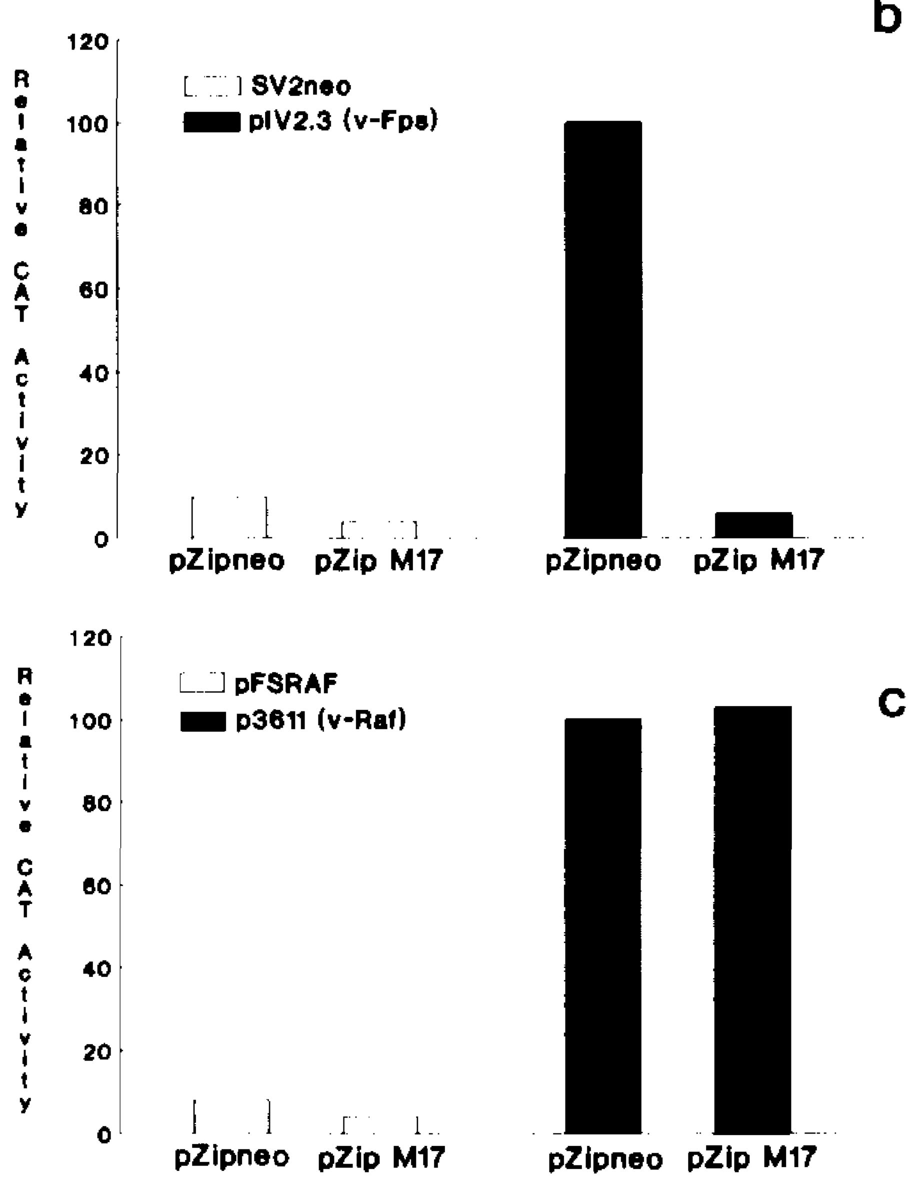


Figure 14. b. Relative CAT activity in the presence of pIV2.3 (v-Fps) or pSV2neo, the parental plasmid for pIV2.3. Values were normalized to the CAT activity observed in the presence of pIV2.3 (v-Fps) which was assigned a value of 100. (S.D. clear bars: pZipneo +/-3; pZip M17 +/-4; hatched bars: pZip M17 +/-2). c. Relative CAT activity in the presence of p3611 which expresses v-Raf or pFSRAF which expresses a deletion mutant of v-Raf. Values were normalized to the CAT activity observed in the presence of p3611 which was assigned a value of 100. (S.D. clear bars: pZipneo +/-2; pZip M17 +/-2; solid bars: pZip M17 +/-10).

A dominant inhibitory mutant of Raf-1 blocks both v-Fps- and v-Ras-induced Egr-1 promoter activation. If Raf-1 functions downstream from HaRas in v-Fps-induced Egr-1 expression, then blocking Raf-1 function would be expected to block both v-Fps and v-HaRas-induced Egr-1 promoter activation. To block Raf-1 expression, we used a recently characterized inhibitory mutant of Raf-1 that was shown to inhibit serum and phorbol ester induced DNA synthesis (Kolch et al., 1991) and v-Src-induced Egr-1 expression (Qureshi et al., 1991). This mutant contains a tryptophan substituted for leucine in the ATP binding site and results in a kinase-defective Raf-1 protein that functions as a dominant inhibitory mutant (Kolch et al., 1991). p301-1 expresses the kinase-defective mutant of Raf-1 under the control of the cytomegalovirus early promoter (Fig. 14a) (Kolch et al., 1991). Cotransfection of p301-1 with pIV2.3 (v-Fps) into NIH 3T3 cells blocked v-Fps induced Egr-1 promoter activation (Fig. 14b). When pv-HaRas was cotransfected in cells with p301-1, a similar decrease in CAT activity was observed (Fig 14c). To demonstrate that the effect of the Raf-1 mutant was not blocking Egr-1 promoter activation, we examined the effect of this mutant on Egr-1 promoter activation induced by the serine/threonine kinase v-Mos (Blair et al., 1980). p301-1 had no effect on CAT gene expression induced by cotransfection of pEgr-1 P1.2 with pHT25 Fig. 14d), which expresses v-Mos (Blair et al., 1980). Thus, the effect of the inhibitory Raf-1 mutant on v-Fps- and v-HaRas-induced Egr-1 promoter activation is not a non-specific effect of the Raf-1 mutant. These data suggest a Raf-1 requirement for both v-Fps and v-HaRas to induce Egr-1 promoter activation (Fig. 16).

DISCUSSION

In this study, we have shown that the protein-tyrosine kinase v-Fps

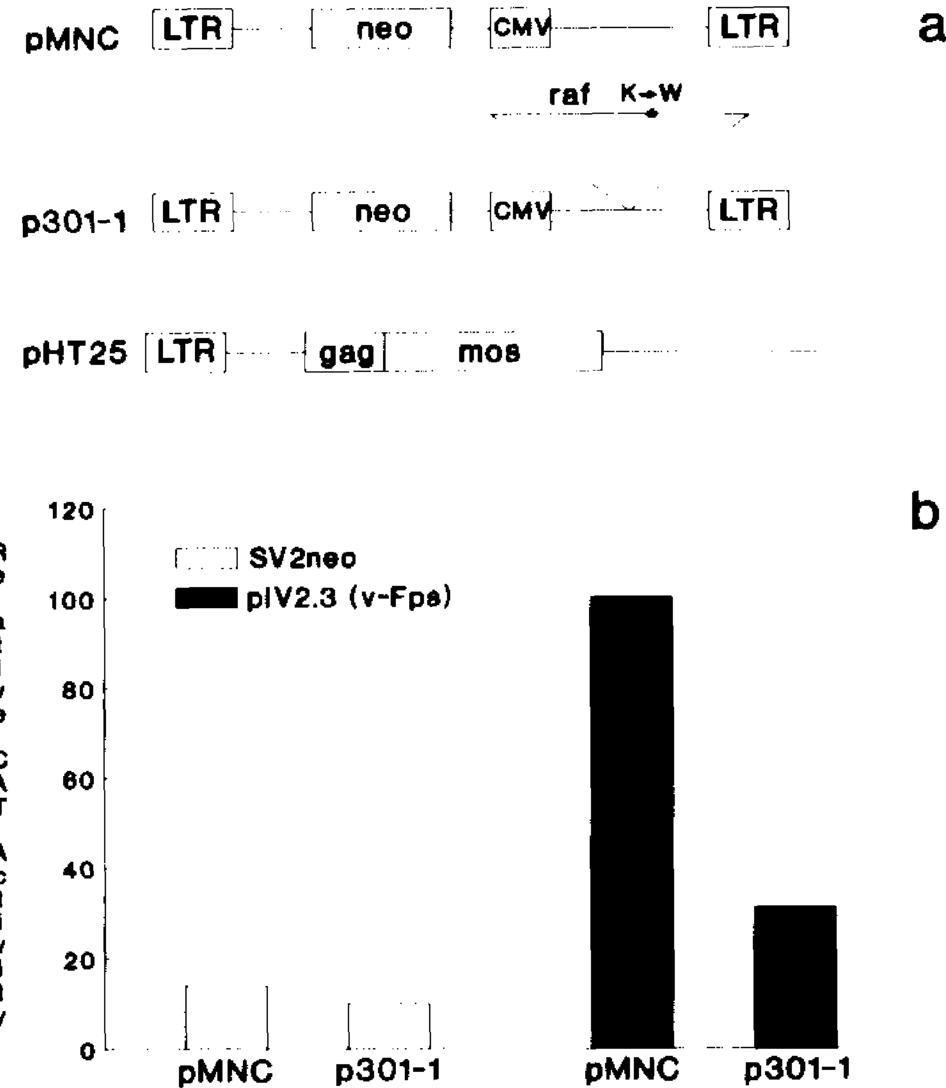
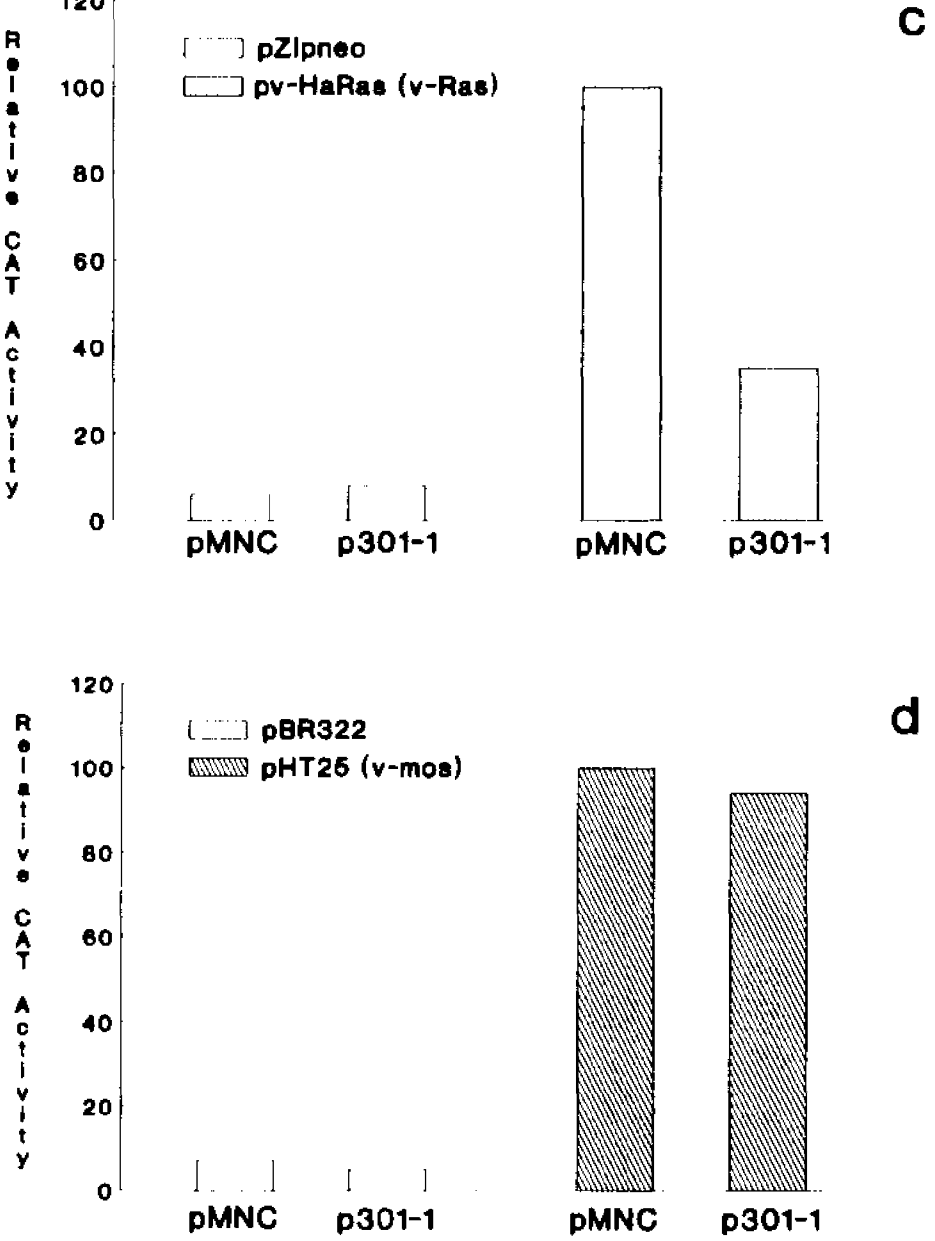


Figure 15. A dominant inhibitory mutant of Raf-1 blocks both v-Fps- and v-HaRas-induced Egr-1 promoter activation. NIH 3T3 cells were transfected with either p301-1, the vector expressing the dominant inhibitory kinase-defective mutant of Raf-1, or pMNC, the parental vector of p301-1. a. Expression vectors used. b, c, and d. p3011 or pMNC were added to the reaction mixture (2.0 ug each per transfection) in the presence of either pIV2.3 (v-Fps) (b), pv-Ha-Ras (v-Ras) (c), or pv-mos (v-Mos) (d). Relative CAT activity induced from the activation of the Egr-1 promoter by v-Fps, v-HaRas, or v-Mos was determined in the absence or presence of the kinase-defective Raf-1 mutant. Values are expressed relative to those obtained in the presence of v-Fps, v-Ras, or v-Mos which were assigned a value of 100. (S.D. b) clear bars: pMNC +/-6; p301-1 +/-3; hatched bars: p301-1 +/-6; c) Clear bars: pMNC +/-1; p301-1 +/-2; hatched bars: p301-1 +/-7; d) clear bars: pMNC +/-3; p301-1 +/-3; hatched bars 301-1 +/-5).


c
Relative CAT Activity
120
100
80
60
40
20
0
pZIpneo
pv-HaRas (v-Ras)
pMNC
p301-1
pMNC
p301-1
d
Relative CAT Activity
120
100
80
60
40
20
0
pBR322
pHT25 (v-mos)
pMNC
p301-1
pMNC
p301-1

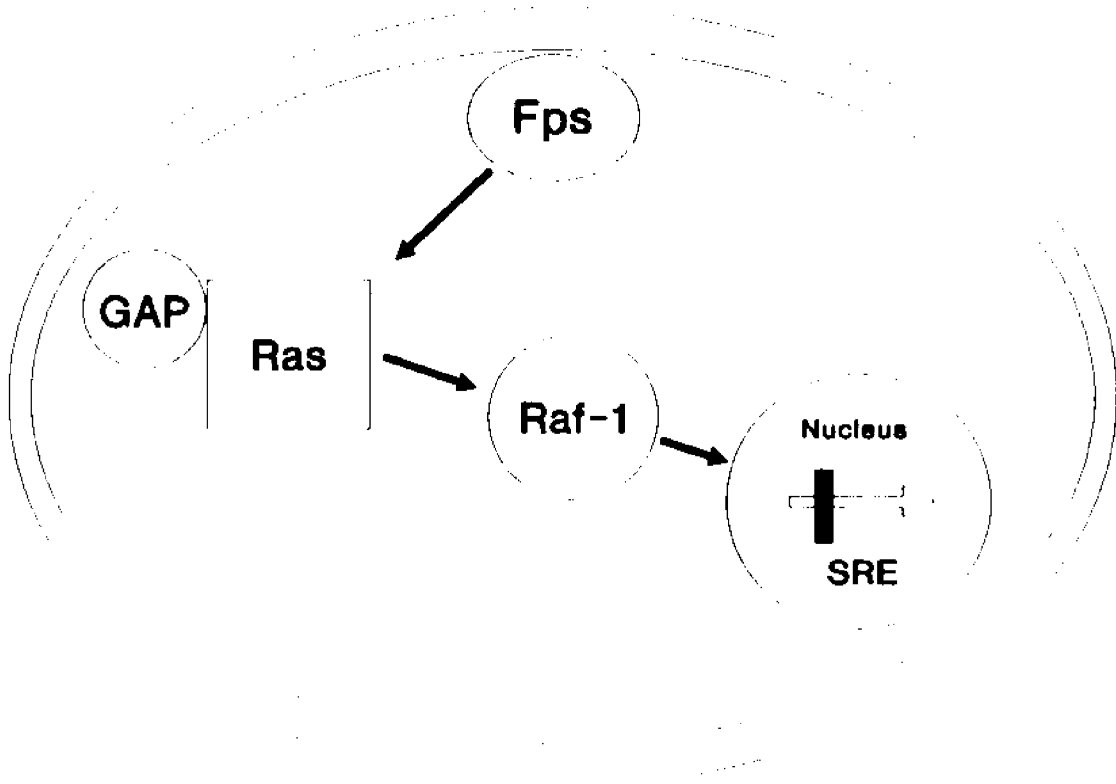


Figure 16. Model for c-Ras and Raf-1 involvement in v-Fps signaling.

transcriptionally activates expression of the mitogen responsive transcription factor Egr-1. The induction of Egr-1 by v-Fps was insensitive to depleting cells of PKC. Activation of the Egr-1 promoter by v-Fps in transient expression assays was sensitive to inhibitory mutants for both HaRas and Raf-1. v-HaRas-induced Egr-1 promoter activation was sensitive to the inhibitory Raf-1 mutant; whereas v-Raf-1-induced Egr-1 promoter activation was unaffected by the M17 HaRas mutant. These data describe an intracellular signal activated by v-Fps that is independent of PKC and dependent upon both HaRas and Raf-1 and where Raf-1 functions downstream of HaRas.

Precise molecular details of HaRas and Raf-1 involvement in v-Fps-induced intracellular signals remain to be determined; however it was recently reported that the GTPase activating protein of HaRas, GAP (Trahey and McCormick, 1987), is phosphorylated on tyrosine in response to v-Fps (9). GAP increases the rate of GTP hydrolysis on HaRas which downregulates HaRas function (Trahey and McCormick, 1987). Thus, GAP could be a target of v-Fps for controlling HaRas activity and the control of HaRas activity could, in turn, contribute to the control of Raf-1 in the HaRas- and Raf-1-dependent intracellular signal characterized here. In this regard, it is of interest that over expression of GAP has been shown to block transformation by v-Src (DeClue et al., 1991) and HaRas (Zhang et al., 1990) - presumably by downregulation of HaRas function. Thus, if the v-Fps-induced-phosphorylation of GAP downregulates GAP activity, this would have the effect of sustaining or upregulating HaRas mediated signals.

Activation of Raf-1 has been reported in response to mitogenic stimulation of quiescent cells (App et al., 1991, Morrison et al., 1989, Morrison et al., 1988, Rapp, 1991). Increased phosphorylation of Raf-1 is believed to be responsible for activating the kinase activity of Raf-1 (Kovancina et al., 1990, Morrison et al., 1988). Raf-1 phosphorylation takes place on serine, threonine and tyrosine residues (Kovancina et al., 1990, Morrison et al., 1988). A mechanism for activation of Raf-1 by v-Fps has

not been determined; however, it has been suggested that the protein tyrosine kinase, v-Src, induces tyrosine phosphorylation on Raf-1 (Morrison et al., 1988). Thus, induction of Raf-1 may involve input by multiple kinases with specificity for both tyrosine and serine/threonine. Since, v-HaRas-induced Egr-1 promoter activation was blocked by the dominant inhibitory Raf-1 mutant, HaRas may function to recruit a protein kinase(s) that phosphorylate and activate Raf-1.

It was previously demonstrated that microinjected antibodies raised against Ras blocked transformation-related phenotypes induced by v-Fps but not by v-Raf (Huleihel et al. 1986, Smith et al., 1986). Thus, the induction of Egr-1 by v-Fps may contribute to the transformed phenotype induced by v-Fps. The report that the tumor suppressing Wilms' tumor gene product binds to the same DNA target sequences as the Egr-1 gene product (Madden et al., 1991, Rausher et al 1990) suggests the possibility of a role for Egr-1 in v-Fps-induced transformation. Thus, the PKC-independent signaling pathway mediated by the protooncogenes HaRas and Raf-1 may prove to be important for transformation induced by v-Fps and perhaps other transforming protein-tyrosine kinases.

PART FOUR: DIFFERENTIAL SENSITIVITY OF V-FPS AND PHORBOL ESTER-INDUCED GENE EXPRESSION TO A DOMINANT NEGATIVE C-HARAS MUTANT

INTRODUCTION

We have demonstrated that activating the protein tyrosine kinase activity of a temperature sensitive derivative of v-Fps activated two distinct intracellular pathways: 1) v-Fps induced the expression of 9E3 via a PKC-dependent signaling mechanism (Spangler et al., 1989,) requiring a GTP-binding protein (Alexandropoulos et al., 1991), and 2) v-Fps induced the expression of the transcription factor Egr-1 through a PKC-independent pathway requiring c-Ras and c-Raf-1 kinase (Alexandropoulos et al., 1992). Thus, v-Fps activates at least two distinguishable intracellular signals that result in the induction of gene expression. The mechanisms through which non-receptor tyrosine kinases activate multiple signals have not been well established. It has been suggested recently that intermolecular contacts between proteins phosphorylated on tyrosines and the src homology domains (SH2) of v-Src, v-Fps, PLCτ, and the GTPase activating protein (GAP) results in the initiation of intracellular signals (reviewed by Cantley et al., 1991). In particular, GAP, the protein that stimulates the GTPase activity of Ras (Trahey and McCormick, 1987), has been shown to be phosphorylated on tyrosine residues in cells transformed by v-Src and v-Fps (Ellis et al., 1990), and to be physically associated with v-Src (Brott et al., 1991). Although the functional significance of GAP phosphorylation and its effect on Ras has not been worked out, Ras plays a critical role in mediating the mitogenic actions of activated tyrosine kinases. In addition to mediating v-src- (Cai et al., 1990, Qureshi et al., 1992) and v-Fps- (Alexandropoulos et al., 1992) induced gene expression, Ras has been shown to be activated by growth factors (Gibbs et al., 1990, Satoh et al., 1990), to be required for nerve growth factor (NGF) differentiation of PC12 cells (Hagag et al., 1986,

Szeberenyi et al., 1990, Kremer et al., 1991), as well as for growth and transformation of NIH 3T3 cells by activated tyrosine kinases (Mulcahy et al., 1985, Smith et al., 1986, Feig and Cooper, 1988).

Although Ras has been implicated in widely ranging physiological responses, the exact mechanism of activation and the nature of the cellular pathways regulated by Ras are not well understood. Existing evidence suggests that Ras functions downstream of receptor and nonreceptor tyrosine kinases (Cai et al., 1990, Szeberenyi et al., 1990, Mulcahy et al., 1985, Kremer et al., 1991, Hagag et al., 1986), and upstream from the serine/threonine kinase c-Raf-1 (reviewed by Rapp,1991, Kolch et al., 1991, Alexandropoulos et al., 1992, Mulcahy et al., 1985). In addition, TPA-induced mitogenesis appears to require Ras (Mulcahy et al., 1985), suggesting that Ras acts downstream from PKC. However, Ras can also activate PKC through phospholipid hydrolysis (Fleisman et al., 1986, Lacal et al., 1987a, 1987b, Pan and Cooper 1990, Wolfman and Macara 1987, Preiss et al., 1986) which places Ras upstream of PKC.

We have shown previously that v-Fps-induced expression of a reporter gene (9E3), is mediated by a G-protein that functions upstream of PKC in CEF. We have also shown that Ras is involved in induction of Egr-1 gene expression in response to v-Fps, independently of PKC. In this study, we have used the Ras inhibitor described above to characterize Ras involvement in two different intracellular pathways initiated by v-Fps, and PKC depletion to characterize the position of Ras relative to PKC in v-Fps induced signaling (Fig.17).

RESULTS

v-Fps activates TRE- and SRE-mediated gene expression. We have previously shown that induction of the protein tyrosine kinase activity of v-Fps leads to

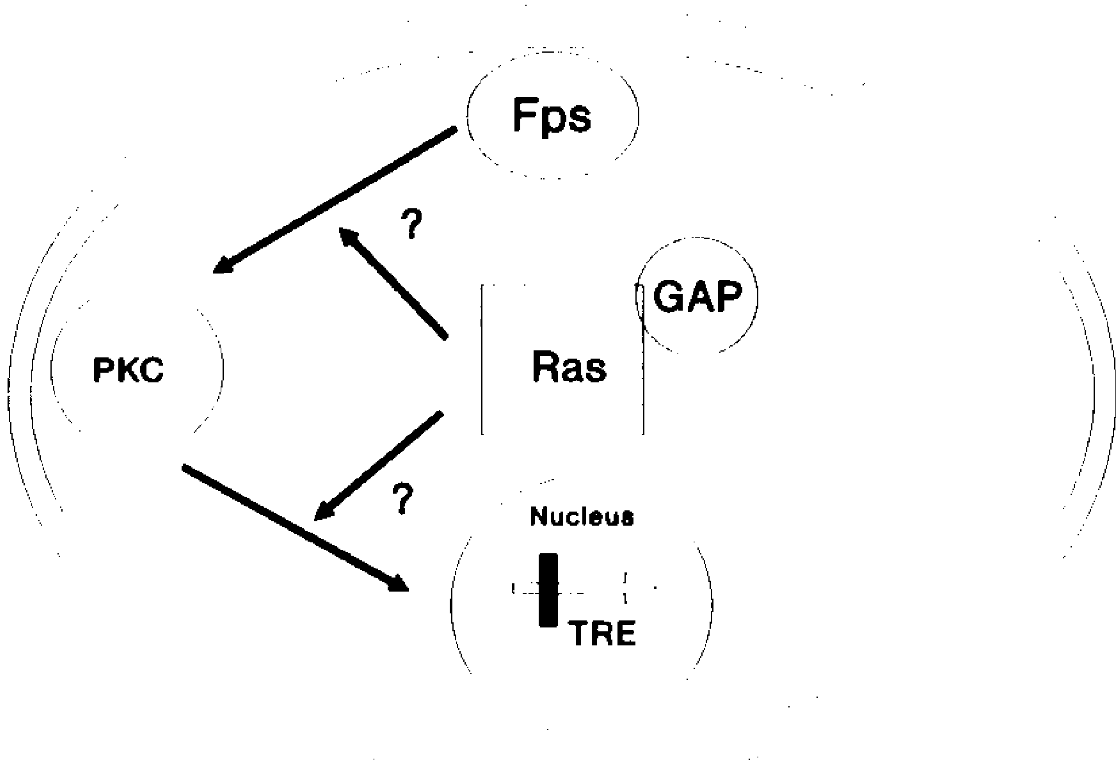
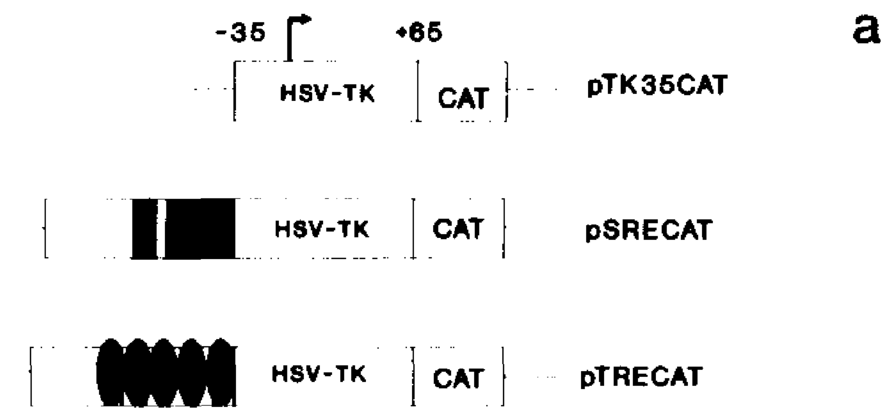


Figure 17. Model for Ras involvement in PKC-dependent pathway.

intracellular signals that are both PKC-dependent (Spangler et al., 1989) and PKC-independent (Alexandropoulos et al., 1992). To further characterize the intracellular pathways induced by v-Fps, we first examined the ability of v-Fps to induce promoter elements known to be activated in a PKC-dependent or PKC-independent manner in transient transfection assays. pTRECAT (Fig. 18a) is activated in response to phorbol esters that activate PKC. Induction is mediated by five tandemly repeated AP-1 sites from the collagenase promoter inserted upstream of the TK promoter fused to the CAT gene (Angel et al., 1987, Fig 18a). v-Fps, when cotransfected with pTRECAT into NIH 3T3 cells, led to activation of TRE-mediated gene expression (Fig. 18b).

pSRECAT (Fig. 18a) contains a cluster of SREs from the Egr-1 gene promoter that has been shown to be responsive to v-Src (Qureshi et al., 1991c) and v-Fps (Alexandropoulos et al., 1992). Cotransfection of v-Fps and pSRECAT, also activated SRE-mediated transcription, (Fig. 18b). The effect of v-Fps on pTRECAT and pSRECAT was due to the presence of the promoter elements since cotransfection of the parental plasmid pTK35CAT (Fig. 18a) for both pSRECAT and pTRECAT with v-Fps, had no effect on transcription (Fig 18b). v-Fps expression had no effect on the basal pTK35CAT promoter activity, nor did expression of SV2neo, the parental plasmid of v-Fps, effect basal CAT expression levels when cotransfected with either pTRECAT or pSRECAT (Fig. 18b).

__c-Ras mediates distinguishable intracellular signals initiated by v-Fps__. We have shown that c-HaRas mediates the activation of the Egr-1 promoter by v-Fps, using a negative inhibitor of c-HaRas (Fig. 13a, 13b) (Alexandropoulos et al., 1992). To further characterize the involvement of c-Ras in v-Fps signaling we tested the ability of the c-Ras mutant to inhibit TRE- and SRE-mediated gene expression initiated in response to v-Fps. pTRECAT and pSRECAT activation by v-Fps were both sensitive to the dominant inhibitor of c-Ras (Fig. 19a). Cotransfection of pTRECAT and



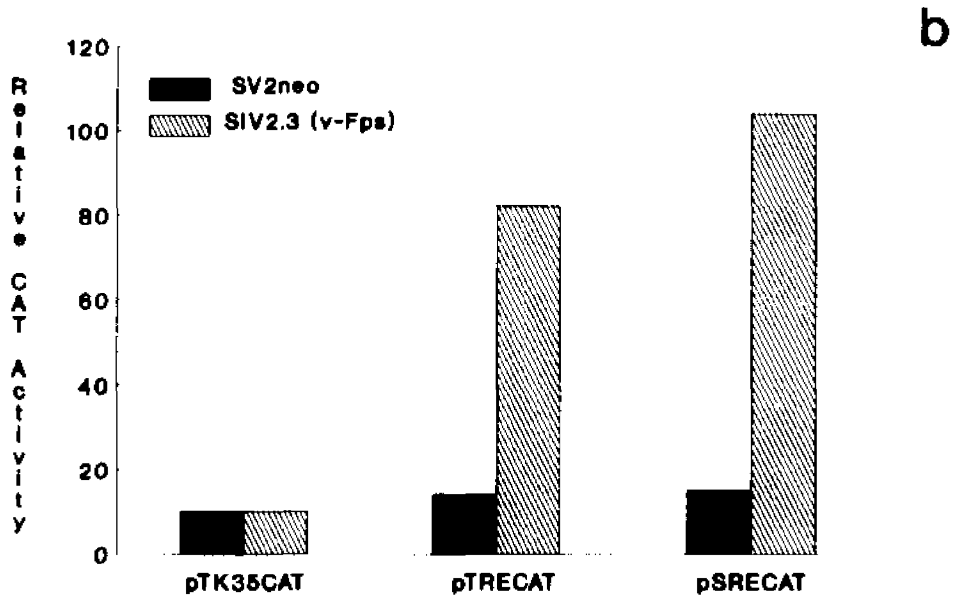


Figure 18. v-Fps activates TRE- and SRE-mediated transcription. NIH 3T3 cells were transfected with three different reporter plasmids expressing the bacterial CAT gene under the control of the HSV TK minimal promoter, the TRE of the collagenase gene, and the SRE of the Egr-1 promoter. pIV2.3, the vector expressing v-Fps, or SV2neo, the parental plasmid for pIV2.3, were included in the transfection mixture. CAT activity was determined as described in Materials and Methods. a. Diagrams of expression vectors used. b. Relative CAT activity induced by v-Fps. Values were normalized to the CAT activity generated in the presence of pSV2neo and pTK35CAT. Data are the mean of at least four independent experiments. (S.D. pTRECAT solid bar: +/-5; hatched bar +/-12; pSRECAT solid bar: +/-3; hatched bar +/-15).

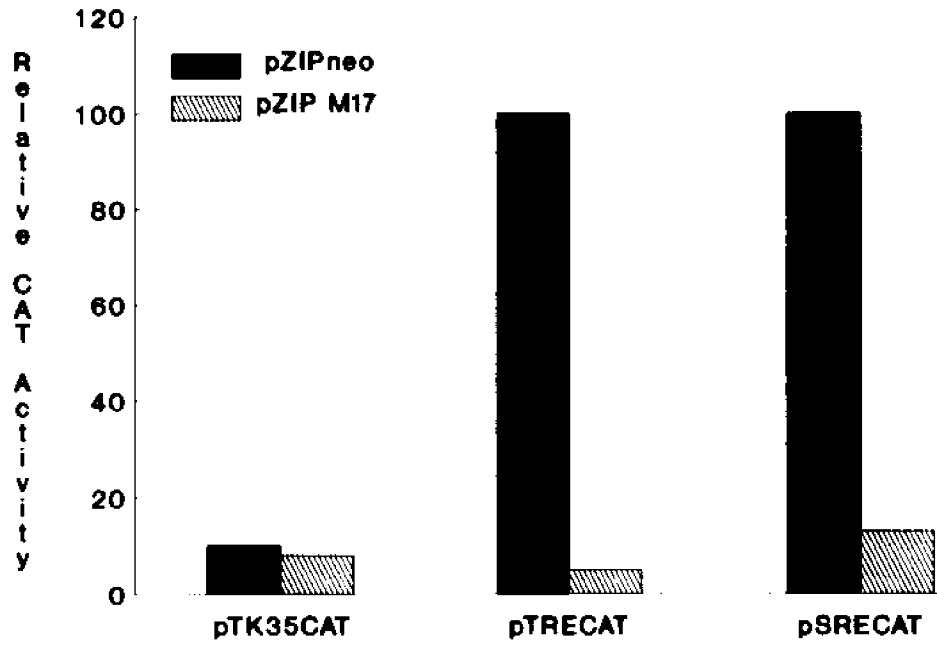


Figure 19. Activation of TRE and SRE by v-Fps is mediated by Ras. a. pTK35CAT, pTRECAT, and pSRECAT were cotransfected with v-Fps into NIH 3T3 cells in the presence of pZIP M17, or the parental vector for pZIP M17, pZIPneoSV(X). Data were normalized to the CAT activity generated in the presence of v-Fps and pZIPneoSV(X). Data are the mean of at least four experiments. (S.D. pTK35CAT solid bar: +/-4; hatched bar: +/-6; pTRECAT hatched bar: +/-4; pSRECAT hatched bar +/-6).

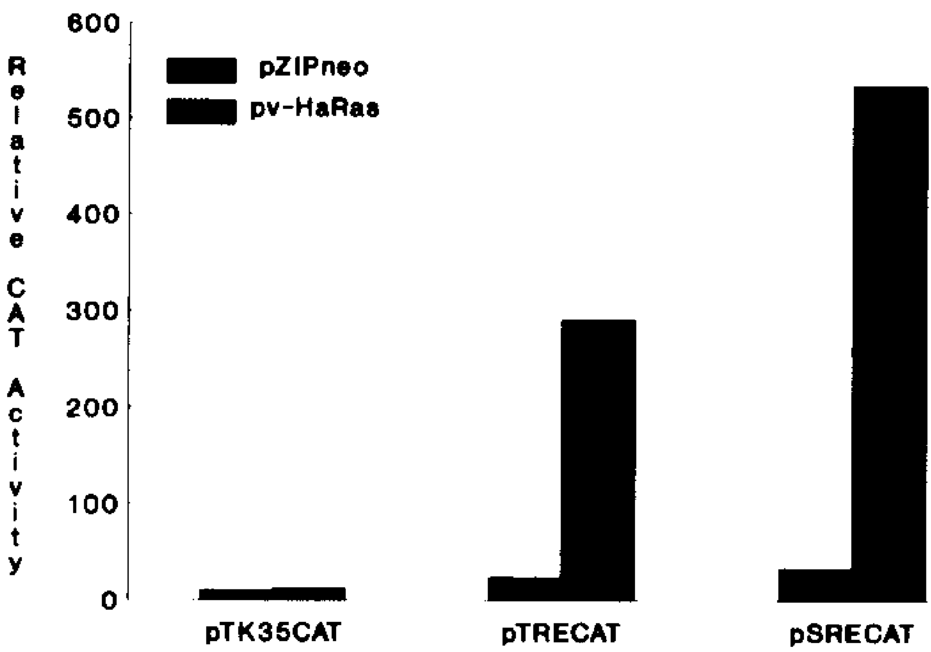


Figure 19. b. Cells were transfected with pTK35CAT, pTRECAT, or pSRECAT in the presence of either pZIPneoSV(X), or pv-HaRas, a vector expressing an activated form of Ras. Data were normalized to the CAT activity generated in the presence of pZIPneoSV(X). Data are the mean of at least four experiments. (S.D. pTK35CAT hatched bar: +/-2; pTRECAT solid bar: +/-4; hatched bar: +/-26; pSRECAT solid bar: +/-7; hatched bar: +/-54).

pSRECAT with the parental vector of pZIP M17 in the presence of v-Fps had no effect on v-Fps promoter activation (Fig. 19a). Therefore, c-Ras can mediate distinguishable intracellular pathways that are initiated by the tyrosine kinase activity of v-Fps.

Consistent with c-Ras involvement implicated by the Ras mutant, v-Ras also induced gene expression in response to TRE and SRE activation. Cotransfection of NIH 3T3 cells with a vector expressing v-Ras (Fig. 13a) along with either pTRECAT or pSRECAT resulted in large increases in CAT activity over that observed with the parental vector pZIPneoSV(X) (Fig. 19b). Therefore, v-Ras can induce gene expression through activation of TREs and SREs.

v-Ras activation of TRE- and SRE-mediated gene expression is insensitive to PKC depletion. We have previously depleted cells of PKC, by prolonged exposure to TPA, to implicate PKC in intracellular signaling (Spangler et al., 1989; Qureshi et al., 1991a; Joseph et al., 1992). To examine where in the pathway with respect to PKC is c-Ras acting, cells were depleted of PKC by exposure to 100 ng/ml TPA for 26 h prior to transfection. The presence of PKC was then examined by western blot analysis using an antibody raised against the conserved catalytic domain of PKC (Ballester and Rosen 1987) as previously described (Joseph et al., 1992). PKC was not detected on western blots after treatment with phorbol esters (data not shown). Depletion of NIH 3T3 cells of PKC, inhibited pTRECAT but not pSRECAT activation by v-Fps (Fig. 20a). However, the v-Ras activation of both TRE- and SRE-mediated transcription were both insensitive to PKC depletion (Fig. 20b), suggesting that Ras functions downstream from or independently of PKC in v-Fps-induced signaling.

TPA-induced TRE- but not SRE-mediated gene expression is sensitive to the Ras dominant inhibitor. To address the question of whether Ras is acting downstream from and/or independently of PKC, NIH 3T3 cells transfected with the reporter plasmids (pTRECAT or pSRECAT) and either pZIPneoSV(X) or pZIP M17 and induced with TPA for 4 h. Addition of TPA to the cells resulted in activation of

reporter plasmids (pTRECAT or pSRECAT) and either pZIPneoSV(X) or pZIP M17 and induced with TPA for 4 h. Addition of TPA to the cells resulted in activation of both pTRECAT and pSRECAT (Fig. 21). TPA-induced TRE activation was inhibited by the c-HaRas mutant, consistent with Ras acting downstream from PKC (Fig. 21); however, SRE activation by TPA was not effected by the Ras mutant (Fig. 21). Therefore, the data suggests that Ras can act both downstream from and independently of PKC in intracellular signaling initiated by v-Fps.

DISCUSSION

We have shown that the nonreceptor tyrosine kinase v-Fps can induce two intracellular signals that lead to increases in gene expression. Induction of both PKC-dependent and PKC-independent gene expression in response to v-Fps is mediated by c-HaRas; thus Ras can function as a transducer of two distinguishable intracellular signaling pathways. In addition, in the PKC-dependent pathway Ras is acting downstream of PKC.

PKC is activated by diacylglycerols (Nishizuka 1988), and we have previously demonstrated that diacylglycerols produced in response to the tyrosine kinase v-Src were generated by a phospholipase D-mediated hydrolysis of phosphatidylcholine (Song et al., 1991). Phospholypase D activity generated in response to v-Src is sensitive to GDPßS in permeabilized mouse fibroblasts (preliminary data). v-Fps also induces diacylglycerol production in rat fibroblasts expressing a ts derivative of v-Fps (unpublished results). Since a G-protein is acting upstream of PKC is v-Fps signaling (Alexandropoulos et al., 1991), it is possible that a heterotrimeric G-protein-phospholipase D coupling mechanism is activating PKC in v-Fps signaling. Consistent with this, the activation of phospholipase D activity has been reported to be dependent upon a G-protein (Bocckino et al., 1987; Hurst et al., 1990; Olson et al., 1991; Xie

and Dubyak, 1991); however, the activation of phospholipase D activity in isolated membranes was unaffected by an antibody to Ras proteins that blocks Ras function. Thus the G-protein involved in the activation of phospholipase D activity is not believed to be a Ras protein (Hurst et al., 1990). Consistent with this, evidence presented in this study suggests that Ras is an effector acting downstream of PKC and phospholipid hydrolysis.

The precise molecular mechanisms for activating HaRas by v-Fps are not well understood. There are two mechanisms through which HaRas signaling is controlled. HaRas is activated by the exchange of GDP for GTP and inactivated by hydrolysis of bound GTP to GDP (reviewed by Bourne 1991). Regulating HaRas activity could then occur by control of either GDP/GTP exchange or GTPase activity. The regulation of HaRas activity by GDP/GTP exchange has not been well characterized. GDP/GTP exchange proteins have been isolated (Wolfman and Macara, 1990; West et al., 1990), but a role for a GDP/GTP exchange protein in tyrosine kinase-induced intracellular signaling has not yet been reported. The GTPase activity of HaRas is regulated by the GTPase activating protein GAP (Trahey and McCormick, 1987). Over expression of GAP blocks v-HaRas (Zhang et al., 1990) and v-Src-induced transformation (Nori, et al., 1991; DeClue et al., 1991), and TPA-induced activation of the p42 mitogen-activated protein (MAP) kinase (Nori et al., 1991; 1992). GAP has also been shown to be phosphorylated on tyrosine in v-Src and v-Fps transformed cells (Ellis et al., 1990). Therefore, v-Fps may regulate HaRas activity by phosphorylating GAP and thus control the rate at which HaRas is downregulated. PKC activity has been suggested to negatively influence GAP activity (Downward et al., 1990; Nori et al., 1992); thus, the involvement of PKC in v-Fps-induced signals may also contribute to HaRas function. By influencing GAP activity, v-Fps could regulate multiple intracellular signaling mechanisms that are dependent upon Ras. Precise molecular details for the control of

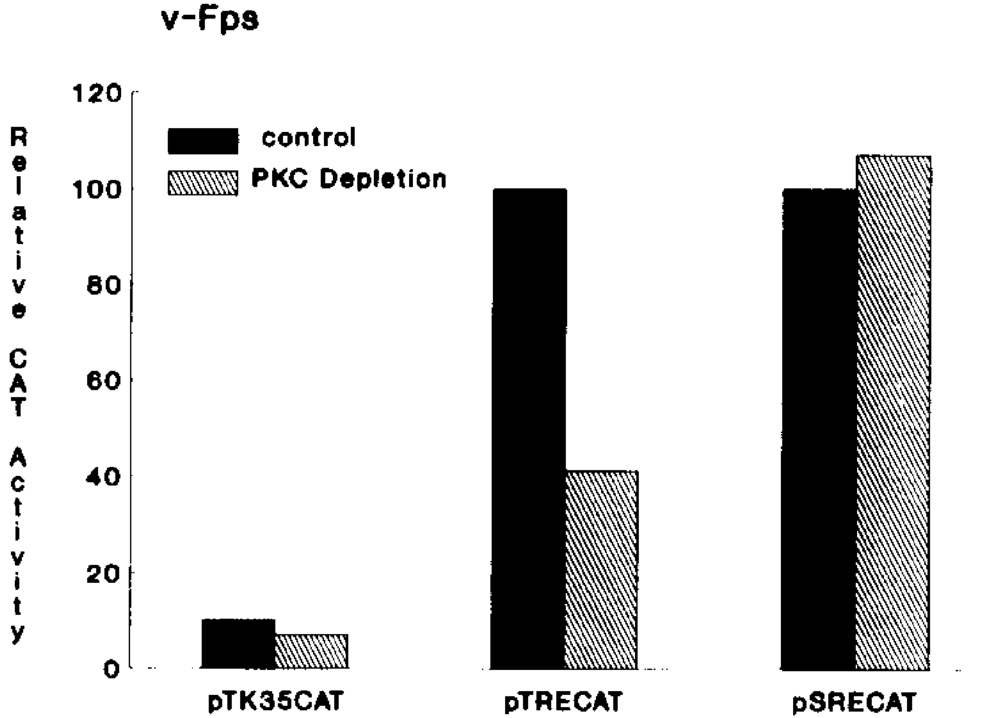


Figure 20. TRE and SRE activation by v-Ras is not sensitive to PKC depletion. To determine if PKC was required for induction of TRE- and SRE-mediated gene expression by v-Ras, cells were depleted of PKC as described in Materials and Methods. a. Depleted cells were cotransfected with the reporter plasmids and v-Fps and data normalized to the CAT activity assayed in extracts of untreated cells. Data are the mean of four independent experiments. (S.D. Hatched bars: pTK35CAT +/-5; pTRECAT +/-9; pSRECAT +/-13).

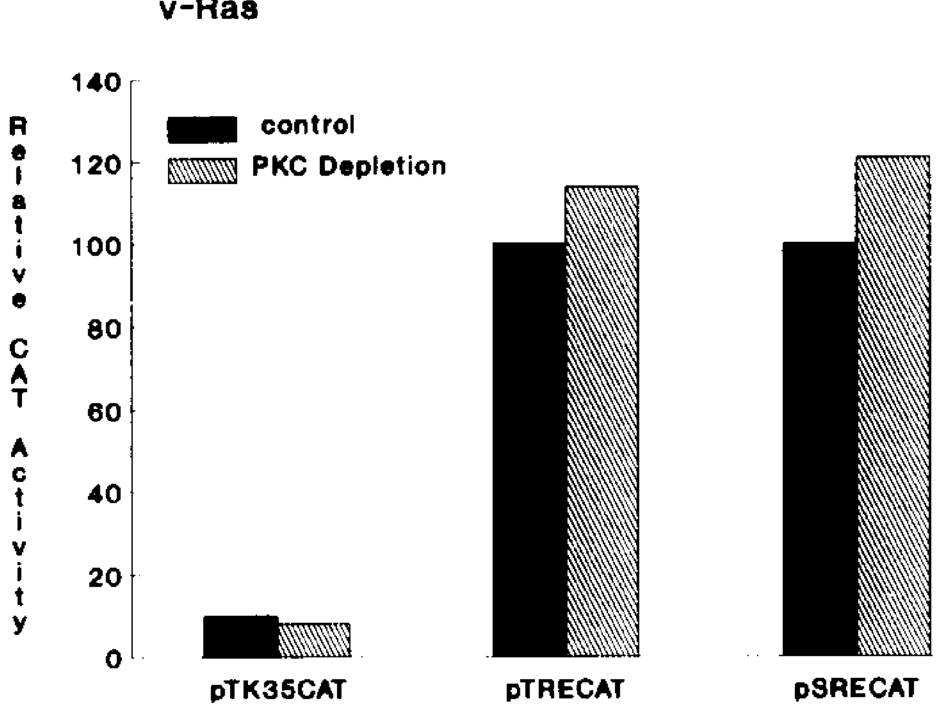


Figure 20. b. Cells depleted of PKC were transfected with pTK35CAT, pTRECAT and pSRECAT in the presence of v-Ras and CAT activity normalized to the activity obtained from extracts of untreated cells. Data are the mean of four independent experiments. (S.D. Hatched bars: pTK35CAT +/-7; pTRECAT +/-17; pSRECAT +/-21).

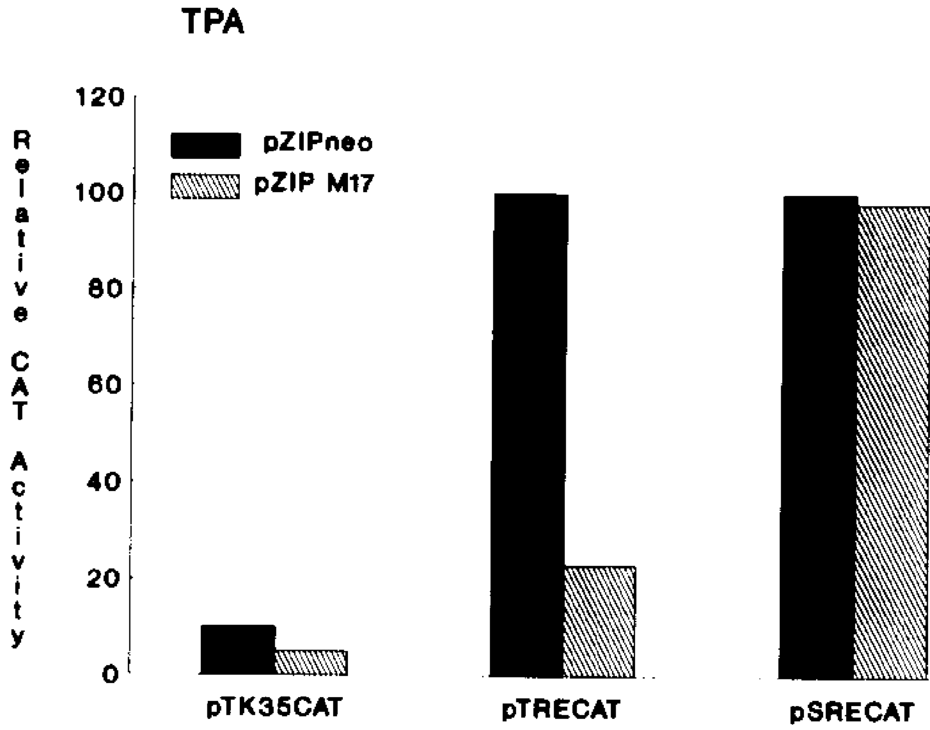


Figure 21. TRE- and SRE-mediated gene expression by TPA are differentially sensitive to the dominant Ras inhibitor. NIH 3T3 cells transfected with the reporter plasmids and either pZIP M17 or its parental vector pZIPneoSV(X) were treated with 100 ng/ml TPA for 4 h prior to harvesting. CAT enzyme activity was assayed and normalized to the activity generated in extracts tr¯ısfected with pZIPneoSV(X). Data are the mean of at least four independent experiments. (S.D. pTK35CAT solid bar: +/-1; hatched bar: +/-4; pTRECAT hatched bar: +/-9; pSRECAT hatched bar: +/-11).

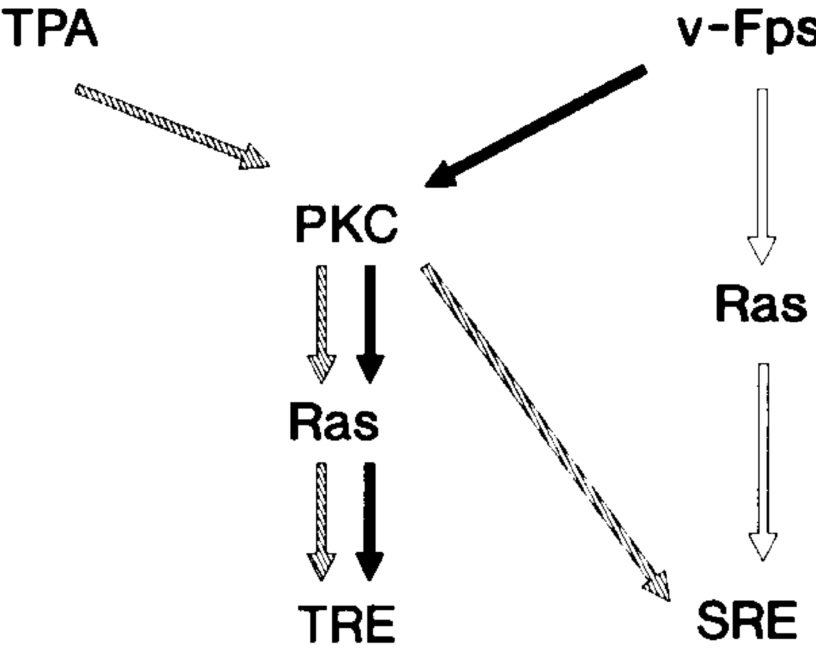


Figure 22. Schematic presentation for c-HaRas involvement for intracellular signals initiated by v-Fps. Ras mediates activation of PKC-dependent and -independent signals initiated by v-Fps, where in PKC-dependent signals Ras acts downstream of PKC. Illustration is highly schematic and does not represent or imply linear pathways or direct interactions between signaling intermediates.

SUMMARY

Protein-tyrosine activity has been implicated as an early event in mitogenic signals. Many receptors for cell division signals are either protein-tyrosine kinases, or are coupled to protein kinases. Mutations that activate the protein tyrosine activity of proteins often lead to a transforming phenotype. The product of Fujinami sarcoma virus v-Fps, is a protein tyrosine kinase that transforms cells in culture and induces tumors in animals. Intracellular signals activated by v-Fps are not well understood. Therefore, in the present study we have examined intracellular components induced in response to activation of the tyrosine kinase activity of the v-Fps oncogene. We have shown that v-Fps signaling mediated by Protein Kinase C, is coupled to a heterotrimeric G-protein that is acting upstream of PKC. v-Fps also activates pathways independently of PKC. Both PKC-dependent and independent signals initiated by v-Fps are mediated by HaRas, a monomeric GTP-binding protein. In PKC-dependent signaling by v-Fps, Ras is functioning downstream of PKC. Raf-1 kinase is also required for PKC-independent signaling in response to v-Fps. Given the existing cellular complexity and the variety of signaling components in mitogenic pathways, it is likely that additional signaling mechanisms are activated by v-Fps that lead to transformation. These novel pathways remain to be determined.

MATERIALS AND METHODS

Materials. GTPγS, GDPβS, ATPγS, phorbol dibutyrate (PBt_2), saponin and digitonin were obtained from Sigma. [γ-^{32}P]-ATP and [a-^{32}P]-dCTP were obtained from New England Nuclear. 8-azido guanosine-5'-triphosphate-[a-^{32}P] was a gift from Boyd E. Haley (University of Kentucky). Cycloheximide, phorbol dibutyrate TPA, and chloramphenicol were obtained from Sigma. (^{14}C)-butyryl-co-enzyme A, (α^{32}P)-dCTP and (α^{32}P)-UTP were obtained from New England Nuclear.

Cells and Viruses. Generation and maintenance of chicken embryo fibroblasts (CEF) and the temperature sensitive derivative of Fujinami sarcoma virus (tsNY225) have been described (Hanafusa et al., 1981). Normal rat 3Y1 cells, and 3Y1 cells transformed by a temperature sensitive derivative of Fujinami Sarcoma Virus NY225 (Hanafusa et al., 1981; Birnbaumer et al., 1987) were maintained in Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum. At the non-permissive temperature (40^{0}C) the cells are non-transformed, where at the permissive temperature (35^{0}C) the cells assume the transformed phenotype. Confluent cell cultures were made quiescent by maintaining the cultures in conditioned medium for four days. NIH 3T3 cells were obtained from the American Type Culture Collection and were also maintained in DMEM supplemented with 10% calf serum.

Permeabilization of CEF and RNA analysis. Permeabilization to allow entry of guanine nucleotides was performed as described by Burch and Axelrod (1987). Cells were incubated in serum-free medium containing saponin (80 ug/ml) for 3 min. Permeabilization was monitored by trypan blue uptake. After permeabilization, cells were rinsed twice with serum-free media lacking saponin but containing guanine nucleotides as indicated, and the cells were further incubated for 90 min. RNA was

then extracted and subjected to northern gel analysis as previously described (Qureshi et al., 1991). Hybridizations were carried out using radiolabeled DNA and washings of the filters were performed as described by Davis et al. (1986).

Analysis of phosphorylated proteins. Permeabilization and analysis of phosphorylated cellular proteins was performed as described by Erusalimsky et al. (1988). Confluent CEF or CEF infected with tsNY225 were washed twice with isotonic buffer (120 mM KCl, 30 mM NaCl, 1mM $MgCl_2$, 1 mM K_2HPO_4, 10 mM sodium PIPES (pH 7.0), 1 mM EGTA and 0.037 mM $CaCl_2$). Cells were then incubated in isotonic buffer containing digitonin (40 ug/ml) and 10 uM [γ-^{32}P]ATP (2 Ci/mmol) for 3 min. After incubation the cells were lysed and subjected to SDS-polyacrylamide gel electrophoresis as previously described by Spangler et al., (1989). Samples were normalized for total protein using the Bio-Rad assay (Bradford, 1976).

Crosslinking of GTP to cellular proteins. CEF or tsNY225-infected CEF were permeabilized for 3 min as described above for the analysis of phosphorylated proteins with permeabilization buffer containing 8-azido-[a-^{32}P]GTP (10 uM, 0.2 Ci/mmol) instead of [γ-^{32}P]ATP. After incubation, cells were UV-crosslinked by irradiation at 4°C with a mineralite UVS-25 at a distance of 2.5 cm for 20 sec as previously described by Northup et al. (1982) and Molina y Vedia et al. (1988). After UV-irradiation, the samples were lysed and subjected to SDS-polyacrylamide gel electrophoresis as described above.

Bacterial toxins. The effect of cholera toxin (obtained from Sigma) was examined over a concentration range of 1 to 20 ug/ml as described previously (Qureshi et al., 1991). Pertussis toxin (also obtained from Sigma) was used over a concentration range of 12 to

100 ng/ml. At the highest concentration (100 ng/ml), pertusis toxin weakly induced 9E3 gene expression. Pertusis toxin was administered 4-6 h prior to temperature shift.

Nuclear "Run-On" Assay. Run-On assays were performed as described by Lineal et al. (1985). Radiolabeled RNA ($2x10^7$ cpm) from 3Y1-NY225 cells was hybridized for 48h to DNA plasmids containing Egr-1 (Lim et al., 1987), murine beta-actin obtained from the American Type Culture Collection and pUC-18 gene sequences that were immobilized on Nylon membrane as described by Marzluff and Huang (1984). Nylon membranes were washed and exposed to X-Ray film for four days with intensifying screens.

Plasmid Constructions. pIV2.3 contains a gag-fps fusion gene cloned into SV_2neo vector (Sadowski et al., 1986). The construction of pEgr-1P1.2, p△Egr-1 P1.2, pE425, p△E425, pE395, pE359, pE342 and pE125 was previously described (Gius et al., 1990, Qureshi et al., 1991c). pE425/250TKCAT was obtained by cloning a Hind III-SmaI fragment from pE425 spanning a region from 425-250 of the Egr-1 promoter, upstream of the HSV-TK promoter of TK35CAT (Jamal and Ziff, 1990). pE395/250TKCAT, pE359/250TKCAT and pE342/250TKCAT were constructed as pE425/250TKCAT using Hind III-SmaI fragments from pE395, pE359 and p342 respectively. pTKCATSRE1-2 was obtained by cloning a Hind III-Hae II fragment from pE425/250TKCAT containing the first and second most distal SREs, into the Hind III-BamHI site of pTK35CAT. pTKCATSRE1,2,3 and 4, constructs contained double stranded oligonucleotides corresponding to the four most distal SREs, were synthesized on an Applied Biosystems oligonucleotide synthesizer. The oligonucleotides were designed to contain the core CArG box along with seven base pairs of the 5'and 3'flanking sequences of each SRE (Tsai-Morris et al., 1988), along with 5'SalI and 3'BamHI restiction sites. The strands were annealed as described (Gius et al., 1990),

and all SREs were cloned into the SalI-BamHI site of pTK35CAT. pZIP M17 contains the Ha-*ras* gene with Asn-17 substituted for Ser-17 in pZIPneoSV(X) (Cao et al., 1990). p301-1 contains a mutated *raf*-1 gene, with the Lysine in the ATP-binding site changed to tryptophan, cloned into pMNC (Kolch et al., 1991). pv-HaRas expresses the v-Ha-*ras* gene in pZIPneoSV(X) (Cao et al., 1990); p3611 expresses a *gag-raf* fusion gene (Jamal and Ziff, 1990), and pHT25 expresses the v-*mos* gene of Moloney sarcoma virus (Blair et al., 1980). pTK35CAT contains the minimal promoter of the thymidine kinase gene of herpes simplex virus (Jamal and Ziff, 1989). pSRETKCAT was constructed by cloning a Hind III-Sma I fragment of the Egr-1 promoter (Tsai-Morris et al., 1989), spanning the region from -425 to -250 relative to the transcriptional start site of the Egr-1 gene, upstream of the herpes simplex virus thymidine kinase promoter in plasmid pTK35CAT (Jamal and Ziff, 1990). This region contains a cluster of four CArG boxes which form the core element of the SREs (Treisman, 1990) and are responsive to v-Fps (Alexandropoulos et al., 1992) and v-Src (Qureshi et al., 1991c). pTRECAT was constructed by cloning a Hind III-Bam HI fragment containing the TRE cassette of p5xTRECAT (Angel et al., 1987) into the Hind III, Bam HI sites of pTK35CAT.

Cell Transfections. NIH 3T3 cells were plated at $5x10^5$ cells in 100mm dishes 20 hours prior to transfection. Transfections were performed using the $CaPO_4$ method as described previously (Qureshi et al., 1991c). Transfection mixtures contained 30 ug of DNA, 5 ug of pIV2.3-fps or SV2-neo, 5 ug of the reporter CAT plasmid and 20 ug of carrier salmon sperm DNA. Transfection mixtures for the Ras and Raf experiments contained 5 μg of inducer or vector DNA, 5 μg of the Ras or the Raf inhibitors (where indicated) and 5 μg of the Egr-1 reporter plasmid. Salmon sperm DNA was added up to 30 μg. Transfection mixtures for the PKC involvement experiments contained 30 μg DNA [5 μg of activator (pIV2.3) or vector (pSV2neo) DNA, 5 μg of reporter CAT

plasmid (pTK35CAT, pSRETKCAT, or pTRETKCAT), 5 μg of the Ras dominant negative inhibitor pZIP M17 or vector (pZIPnoeSV(X)) DNA, and carrier salmon sperm DNA to 30 μg.

CAT-Assay. Transfected cultures were washed 16-20 h post-transfection, and fresh media containing 1% serum was added. After another 30 h, cells were harvested after washing twice with cold isotonic buffer, resuspended in 200 μl og 250 mM Tris-HCL (pH7.8), and lysed by five cycles of freeze-thaw (dry ice/methanol, 37°C) for 5 min each. Supernatants were collected after microcentrifuging for 5 min at 4°C. CAT activity in cell extracts, using equal amounts of protein (Bradford 1976), was determined using the "fluor diffusion assay" (Neuman 1987).

PKC Depletion. Cells were depleted of PKC by treating cells with TPA (100ng/ml) at the time of plating for transient transfection assays. The cells were maintained in 100 ng/ml TPA until the time of harvest for CAT analysis as described above.

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