11. W. H. McClain, C. Guerrier-Takada, S. Altman, unpublished results.
12. Synthetic genes in plasmid pGFIB flanked by Eco RI/Pst I restriction-enzyme sites (Fig. 2) were cloned into plasmid pGEM-2 (Promega Biotec) for in vitro transcription studies. The manufacturer's protocols were used. Briefly, plasmid pGFIB DNA was digested by Eco RI/Pst I, the fragment carrying the synthetic gene was isolated and inserted into the Eco RI/Pst I site of plasmid pGEM-2 DNA. Plasmid pGEM-2 carrying the synthetic gene was digested with Pst I, and the resulting linear DNA was transcribed in vitro by SP6 RNA polymerase. SP6 transcription of this plasmid gives a precursor containing a short 5′ leader sequence, pppGAAUACACGGAAUUC (*13*), and an extra 3′ C residue corresponding to the residual part of the Pst I–digested restriction-enzyme site.
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21. We thank J. Schneider and K. L. Mittelstadt for expert technical assistance; and A. Vioque for the gift of the C5 protein. Research at the University of Wisconsin was supported by PHS grant AI10257 and at Yale University by PHS grant GM19422 and NSF grant DMB8418931.

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Recombinant Fragment of Protein Kinase Inhibitor Blocks Cyclic AMP–Dependent Gene Transcription

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Transcriptional regulation by cyclic adenosine monophosphate (cAMP) in mammalian cells could be mediated by a phosphoprotein substrate of the cAMP-dependent protein kinase or, as in prokaryotes, by a cAMP-binding protein. Two synthetic genes that code for an active fragment of the protein inhibitor of this kinase and a mutant inactive fragment were constructed and used to distinguish these alternatives. Transient expression of the active peptide product specifically inhibited the cAMP-stimulated expression of a cotransfected reporter gene by more than 90 percent, whereas the expression of the inactive peptide did not alter cAMP-stimulated gene expression. The results indicate that an active kinase catalytic subunit is a necessary intermediate in the cAMP stimulation of gene transcription.

Protein kinases and protein phosphatases catalyze a set of reversible posttranslational modifications that are associated with the regulation of many cellular processes (*1, 2*). Certain of these enzymes are subject to inhibition by high-affinity, specific interaction with other proteins, such as the heat-stable inhibitor of the cyclic adenosine monophosphate (cAMP)–dependent protein kinase (PKI, protein kinase inhibitor), first described by Walsh and his colleagues (*3*); PKI is a high-affinity (K_i near 1 n*M*) inhibitor that competes with protein substrates for binding to the free catalytic subunit of the kinase (*3*). Studies with synthetic peptides corresponding to the amino acid sequence of rabbit skeletal muscle PKI have demonstrated the importance of the pseudosubstrate site RRNAI (*4*) (residues 18–22), as well as residues nearer the amino terminus, in conferring the very high affinity and specificity of PKI (*5*). Thus most, perhaps all, of the structural features required for kinase inhibition are located within the NH_2-terminal third of rabbit skeletal muscle PKI, a 75–amino acid protein.

We synthesized a peptide [PKI(1–31), Fig. 1A] corresponding to the NH_2-terminal 31 residues of the rabbit skeletal muscle PKI (*6*). This peptide inhibited competitively (K_i = 4 nM) the phosphorylation of a synthetic peptide substrate (LRRASLG) by the catalytic subunit of cAMP-dependent protein kinase. The specificity of purified PKI(1–75) was maintained by synthetic PKI(1–31). In assays in vitro with purified enzymes, the cyclic guanosine monophosphate–dependent protein kinase, protein kinase C, and protein phosphatase 1 were not inhibited at all by PKI(1–31) at concentrations up to 1.9, 6.1, and 12 μ*M* peptide, respectively. The crucial role of PKI residues 18 and 19 was confirmed by synthesis of PKI peptides in which one or both of these arginine residues had been replaced by glycine; these peptides exhibited greatly diminished ([Gly^{19}]PKI(1–31)) or no detectable PKI activity ([$Gly^{18,19}$]PKI(1–31), Fig. 1B).

A gene coding for an initiator methionine and PKI(1–31) was designed and constructed from synthetic oligonucleotides (*7*) (Fig. 1A); a gene coding for the inactive peptide [$Gly^{18,19}$]PKI(1–31) was prepared by replacing the Sfi I–Bsm I restriction fragment of the PKI(1–31) minigene with mutant oligonucleotides that would replace arginines 18 and 19 in the PKI sequence with glycines. Wild-type PKI(1–31) and mutant [$Gly^{18,19}$]PKI(1–31) peptides were expressed in *Escherichia coli* as lacZ fusion proteins; insoluble inclusion bodies of the parent lacZ product and the fusion proteins were partially purified, treated with CNBr, lyophilized, and reconstituted in neutral buffer. The mixture of CNBr peptides was assayed for PKI content by radioimmunoassay and for protein kinase inhibitory activity. Whereas the CNBr peptides derived from the lacZ-[$Gly^{18,19}$]PKI(1–31) fusion product showed substantial PKI-like immunoreactivity, this preparation gave no inhibition of protein kinase activity (Fig. 1B). In contrast, the CNBr peptides derived from lacZ-PKI(1–31) fusion protein exhibited protein kinase inhibitory activity, which, in relation to the measured content of PKI(1–31), had a comparable or slightly greater potency than that of the synthetic PKI(1–31) peptide (Fig. 1B). Thus the synthetic minigene for PKI(1–31) codes for a functional PKI(1–31) peptide, whereas the mutant minigene [$Gly^{18,19}$]PKI(1–31) is itself functional but codes for a peptide that lacks kinase inhibitory activity.

The synthetic genes were cloned into the eukaryotic expression vector πLXX (*8*) to produce the plasmids πLXX-PKI(1–31) (Fig. 1C) and πLXX-[$Gly^{18,19}$]PKI(1–31). Expression of πLXX-PKI(1–31) was examined after transfection of CV-1 and COS-M6 cells. Hybridization of total RNA with PKI ^{32}P-labeled oligonucleotides revealed a 1.2-kb RNA species in both cell lines, in agreement with the size predicted. S1 nuclease protection assay with a uniformly labeled RNA probe that recognized both πLXX and πLXX-PKI(1–31) messenger RNAs (mRNAs) gave protected fragments of the expected size. Neither the size nor abundance of πLXX or πLXX-PKI(1–31) mRNA was affected by cAMP analogs.

Translation of PKI(1–31) mRNA to produce the recombinant peptide was demonstrated after transfection of COS-M6 cells. Extracts of cells transfected with πLXX or

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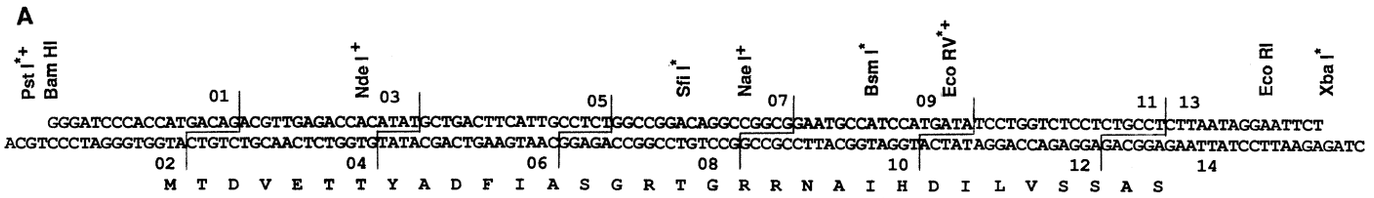

Fig. 1. Construction and expression of PKI(1–31) gene. (**A**) Nucleotide and amino acid sequence of PKI(1–31) gene. Restriction sites marked * or + are unique in pUC12-PKI(1–31) or πLXX-PKI(1–31) (see C), respectively. (**B**) Protein kinase inhibition by synthetic and biosynthetic PKI peptides. Inhibition of cAMP-dependent protein kinase catalytic subunit was determined as in (*5*) using bovine protein kinase catalytic subunit, mixed histone, and various concentrations of synthetic PKI(1–31) (○) and [$Gly^{18,19}$] PKI(1–31) (□) and biosynthetic, CNBr-cleaved lacZ-PKI(1–31) (●) and lacZ-[$Gly^{18,19}$]PKI(1–31) (■). Synthetic PKI peptides were quantified by amino acid analysis. LacZ fusion proteins were generated from a prokaryotic expression vector that produces a truncated β-galactosidase with PKI(1–31) fused to its COOH-terminus. Insoluble fusion proteins were purified, cleaved by CNBr, and quantified by radioimmunoassay; parallel displacement curves of ^{125}I-labeled PKI(1–31) were observed with synthetic peptides and the CNBr digests of the homologous lacZ fusion protein. (**C**) PKI(1–31) minigene in the eukaryotic expression vector πLXX. SV40 ori, eukaryotic origin of replication from SV40; πVX ori, prokaryotic origin of replication from πVX (originally from pMB1); ASV LTR; long terminal repeat from avian sarcoma virus; SV2 intron + A_n; SV40 late splice site and polyadenylation signals from pSV2. The entire Pst I–Xba I PKI gene fragments from both wild-type and mutant constructs were purified and recloned into Pst I–Xba I digested πLXX.

πLXX-PKI(1–31) were analyzed for PKI by radioimmunoassay. PKI immunoreactivity is present in extracts of cells at the time of transfection, presumably as a reflection of endogenous PKI. Transfection with πLXX-PKI(1–31) caused a time-dependent five- to tenfold increase in total PKI immunoreactivity, which was not observed with πLXX. In two experiments, PKI immunoreactivity increased rapidly during the first 24 hours after transfection and began to level off during the subsequent 24 hours.

Because an unknown but probably low percentage of the cells exposed to the πLXX-PKI(1–31) DNA precipitate take up and express the DNA, it was necessary to monitor selectively cAMP responsiveness in that subfraction of cells, in order to assess the effects of PKI(1–31) on cAMP-directed gene transcription. This was accomplished by cotransfection of πLXX-PKI(1–31) with a cAMP-responsive reporter gene; the plasmid pENKAT-12 contains a cAMP-regulated fusion gene consisting of the cAMP-responsive promoter element from the human enkephalin gene and the bacterial chloramphenicol acetyl transferase (CAT) gene. Analogs of cAMP and activators of adenylate cyclase induce a 5- to 20-fold elevation of CAT activity and ENKAT transcript levels in transient expression experiments (*9*). A similar degree of cAMP-stimulated CAT expression was observed when pENKAT-12 was cotransfected with πLXX (Table 1). In contrast, cotransfection of pENKAT-12 with πLXX-PKI(1–31) was associated with a dose-dependent inhibition of the cAMP-stimulated CAT activity (Table 1); in general it reached a plateau at 90 to 95% inhibition (Table 1). Similar results were seen in CV-1 and COS cells (Table 1). This inhibition of cAMP-stimulated CAT activity was paralleled by an inhibition of the cAMP stimulation of correctly initiated pENKAT-12 mRNA, as judged by S1 analysis (Fig. 2).

Table 1. Effects of πLXX vs πLXX-PKI(1–31) on cAMP-stimulated expression of pENKAT-12. CV-1 or COS-M6 cells were plated at 3×10^5 cells per 10-cm tissue culture plate; 18 hours later, cells were transfected with a $CaPO_4$ precipitate containing pENKAT-12 (4 μg/plate), πLXX or πLXX-PKI(1–31) (amounts indicated) and pRSVβgal (10 μg/plate), included as an internal control for variations in transfection efficiency among separate DNA precipitates. The regulators 8-Br-cAMP and 1-methyl-3-isobutylxanthine (IBMX) were added directly to culture medium to 0.5 m*M* each at 24 hours after DNA addition. Cells were harvested 6 to 8 hours later and lysed. CAT assays were performed by a modification of the standard procedure (*12*); briefly, butyryl-CoA is substituted for acetyl-CoA and butyryl-chloramphenicol is selectively extracted into 2,6,10,14-tetramethyl pentadecane and xylene (2:1) with an efficiency of approximately 35%. Data are shown as percentage conversion of [^{14}C]chloramphenicol to butyryl-chloramphenicol normalized for β-galactosidase expression. Percentage inhibition is calculated as follows: the difference in the percentage conversion between treated and untreated cells transfected with πLXX alone was divided into the difference between treated and untreated cells transfected with πLXX-PKI(1–31) alone or as a mixture with πLXX; this fraction was subtracted from 1 and multiplied by 100. Data shown are the average of duplicate plates from a representative experiment.

Cell type	DNA (μg)		CAT activity % conversion 8-Br-cAMP + IBMX		Inhibition by πLXX-PKI(1–31) (%)
	πLXX	πLXX-PKI(1–31)	Untreated	Treated	
CV-1	16	0	0.34	3.69	
	12	4	0.39	2.12	48
	0	16	0.32	0.55	93
COS-M6	7	0	2.61	15.31	
	5.6	1.4	3.69	11.03	42
	0	7	3.04	4.55	88

The specificity of πLXX-PKI(1–31) inhibition of cAMP-regulated transcription is supported by several lines of evidence. πLXX-PKI(1–31) did not inhibit the expression of pENKAT-12 observed in the

absence of added cAMP (Table 1 and Fig. 2), nor the expression of a cotransfected RSV LTR–β-galactosidase fusion gene (pRSVβgal). In addition, when πLXX-PKI(1–31) was cotransfected in a mixture with pENKAT-12 and pXGH5, a plasmid containing a fusion gene consisting of the mouse metallothionein promoter and human growth hormone genes, the cAMP-stimulated expression of the CAT gene was drastically reduced, whereas the Cd^{2+}-stimulated production of human growth hormone remained unaffected by πLXX-PKI(1–31) (Fig. 3). Thus PKI(1–31) inhibits only the component of transcription that is stimulated by elevation of cAMP above basal levels and does not inhibit transcription from constitutive or non–cAMP-regulated promoters.

The strongest evidence that the inhibitory effect of πLXX-PKI(1–31) on cAMP induction of ENKAT transcription is a consequence of the inhibition of the kinase catalytic subunit by the recombinant peptide is provided by comparison with [$Gly^{18,19}$]πLXX-PKI(1–31). This plasmid, which codes for an inactive PKI mutant peptide, failed to provide any inhibition of cAMP-stimulated CAT activity when cotransfected with pENKAT-12 into CV-1 cells (Fig. 4). Thus to inhibit cAMP-stimulated gene transcription, a PKI peptide must be a functional kinase inhibitor.

Transcriptional regulation by cAMP in mammalian cells could be mediated by a phosphoprotein substrate of the cAMP-dependent protein kinase or, as in prokaryotes, by a cAMP-binding protein without a requirement for protein phosphorylation. Our data indicate that expression of πLXX-PKI(1–31), which codes for a specific inhibitor of the catalytic subunit of the cAMP-dependent protein kinase, selectively inhibits cAMP-directed gene expression. We conclude that cAMP-stimulated transcription requires an active kinase catalytic subunit and in turn that a phosphoprotein substrate is required for this response. These results do not eliminate a requirement for a cAMP-binding protein as well but indicate that such a protein by itself cannot sustain cAMP-stimulated transcription. Although the identity of the phosphoprotein substrate required for cAMP-directed transcriptional regulation is unknown, several observations suggest that the phosphoform of the RII isotype of the regulatory subunit of the kinase merits specific consideration (*10*). Moreover, since the levels of the regulatory and catalytic subunits of the kinase are coordinately regulated (*11*), expression of recombinant PKI(1–31) may alter indirectly cellular levels of regulatory subunits. Thus the present results do not exclude P-RII as the necessary phosphoprotein in transcriptional regulation by cAMP. An important assumption that underlies the present study is that recombinant PKI(1–31) retains the high specificity for inhibition of cAMP-directed protein phosphorylation observed

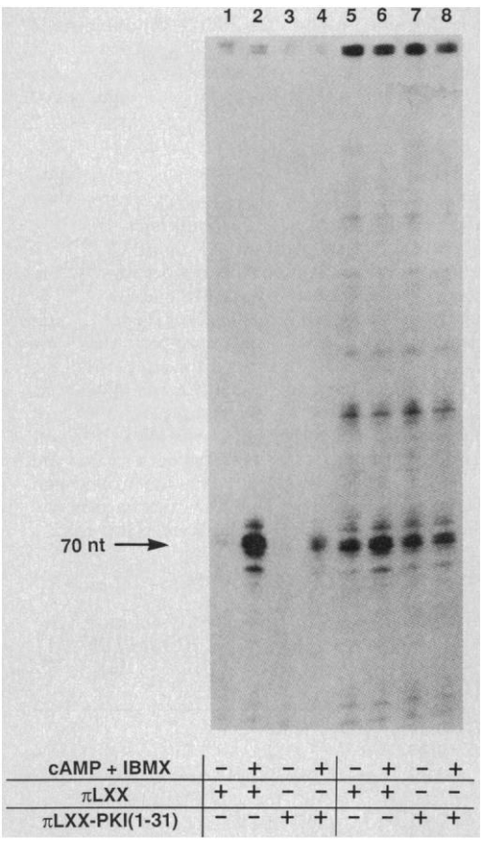

Fig. 2. S1 analysis of pENKAT-12 mRNA. CV-1 cells (lanes 1 to 4) and COS-M6 cells (lanes 5 to 8) were transfected with pENKAT-12 and with πLXX (lanes 1, 2, 5, and 6) or πLXX-PKI(1–31) (lanes 3, 4, 7, and 8) and treated with no addition (lanes 1, 3, 5, and 7) or with 0.5 m*M* 8-Br-cAMP plus 0.5 m*M* IBMX (lanes 2, 4, 6, and 8), as described in the legend to Table 1. Ninety minutes after 8-Br-cAMP + IBMX, the cells were harvested, cytoplasmic RNA was isolated, and S1 analysis was carried out as described (*9*). The protected fragment (70 nucleotides) corresponding to the correctly initiated pENKAT-12 transcript is indicated.

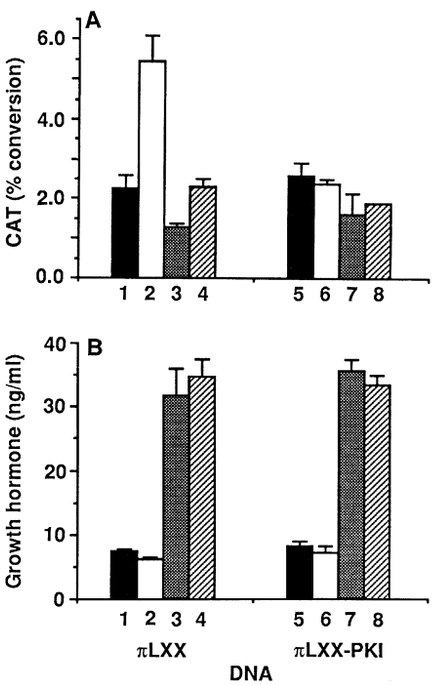

Fig. 3. Comparison of effects of πLXX-PKI(1–31) on gene expression from cAMP- and metal-regulated promoters. (**A**) CAT activity was calculated as described in the legend to Table 1. (**B**) Concentration of human growth hormone in culture media. Extracts from πLXX-transfected (bars 1 to 4) and πLXX-PKI(1–31) transfected (bars 5 to 8) cells treated with no additions (bars 1 and 5), 8-Br-cAMP and IBMX (0.5 m*M* each, bars 2 and 6), $CdCl_2$ (2.6 μ*M*, bars 3 and 7), or 8-Br-cAMP, IBMX, and $CdCl_2$ (bars 4 and 8). CV-1 cells were transfected as described in the legend to Table 1, except that the $CaPO_4$ precipitate contained 4 μg of pENKAT-12, 10 μg of pRSVβgal, 10 μg of πLXX or πLXX-PKI(1–31), and 2.5 μg of pXGH5 (*13*) a mouse metallothionein promoter–human growth hormone gene fusion. $CdCl_2$ was added 17 hours after glycerol shock, and 8-Br-cAMP and IBMX were added 47 hours after glycerol shock; cells were collected 14 hours later. CAT activity was assayed as described in the legend to Table 1, and human growth hormone in the culture media was measured by radioimmunoassay (Hybritech). The bars indicate the range of values observed on duplicate plates. Results shown are representative of three experiments.

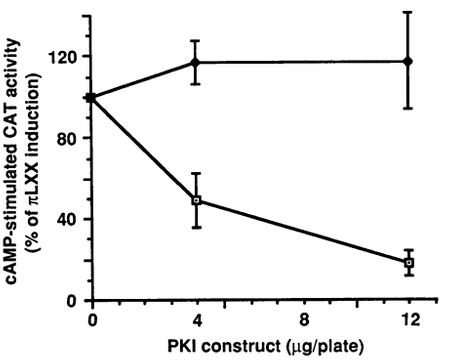

Fig. 4. Effects of wild-type and mutant PKI on cAMP-stimulated CAT activity. CAT activity in extracts of CV-1 cells (two experiments) and COS cells (three experiments) transfected with ENKAT and πLXX-PKI(1–31) (□) or ENKAT and πLXX-[$Gly^{18,19}$]PKI(1–31) (◆) and treated with 8-Br-cAMP + IBMX is plotted as a percentage of the CAT activity measured in extracts of cells transfected with ENKAT and πLXX (■) and treated with 8-Br-cAMP + IBMX. Cells were transfected, extracted, and assayed as described in the legend to Table 1, except that the $CaPO_4$ precipitate contained 5 μg of pENKAT-12, 10 μg of pRSVβgal, and a total of 12 μg of πLXX-derived DNA, either as πLXX alone (■) or with πLXX-PKI(1–31), 4 or 12 μg (□); or πLXX-[$Gly^{18,19}$]PKI(1–31), 4 or 12 μg (◆). The bars indicate standard error of the mean; inhibition by πLXX-PKI(1–31) is significant ($P < 0.01$) relative to πLXX-[$Gly^{18,19}$]PKI(1–31) at the corresponding dose and to πLXX.

for the synthetic peptide. Direct validation of this point will require regulated expression of PKI(1–31) in most or all the cells, so as to permit direct examination of the endogenous (intracellular) protein phosphorylation in the presence and absence of this peptide. The present data, however, support the specificity of the effects of PKI(1–31) on cAMP-directed transcriptional regulation and furthermore indicate the feasibility of introducing bioactive peptide fragments intracellularly via recombinant DNA techniques.

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4. The one-letter symbols used for amino acids are as follows: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; N, asparagine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine; and X, unspecified.
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7. The published rabbit skeletal muscle PKI sequence (*6*) was back-translated according to R. Lathe [*J. Mol. Biol.* **182**, 1 (1985)] by means of a codon frequency table generated from 40 arbitrarily chosen rabbit genes. The consensus CCACC [M. Kozak, *Nucleic Acids Res.* **12**, 857 (1984)] and a methionine initiator codon were added at the amino terminus of the coding region, and two termination codons were added at the carboxyl terminus. The gene was assembled from 14 oligonucleotides (boundaries are indicated) as described by E. L. Brown, R. Belagaje, M. J. Ryan, and H. G. Khorana [*Methods Enzymol.* **68**, 109 (1979)], cloned in pUC12, and confirmed by DNA sequencing.
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14. We thank M. Comb, N. Birnberg, B. Seed, S. Aruffo, and D. Moore for helpful discussions; M. Comb, S. Aruffo, and D. Moore for gifts of plasmids pENKAT-12, πLXX, and pXGH5, respectively; J. Smith for initial synthesis of PKI(1–31); G. Curtis for tissue culture; I. Kang and M. Fitzgibbon for technical assistance; and M. Chambers for typing the manuscript. J. Corbin provided purified cGMP-dependent protein kinase. W. Wetekam (Hoechst AG) provided the β-galactosidase vector described in Fig. 1 as a gift. Supported in part by a grant from Hoechst AG.

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Rapid Stimulation of Diacylglycerol Production in *Xenopus* Oocytes by Microinjection of H-*ras* p21

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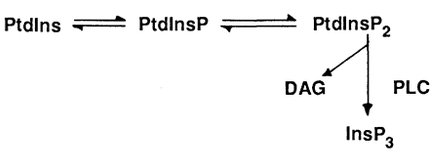
The p21 products of *ras* proto-oncogenes are thought to be important components in pathways regulating normal cell proliferation and differentiation. These proteins acquire transforming properties as a result of activating lesions that convert *ras* genes to oncogenes in a wide array of malignancies. In *Xenopus laevis* oocytes, microinjection of transforming *ras* p21 is a potent inducer of maturation, whereas microinjection of a monoclonal antibody to *ras* p21 inhibits normal maturation induced by hormones. The phosphoinositide pathway is a ubiquitous system that appears to play a key role in diverse cellular functions. By use of the *Xenopus* oocyte system, it was possible to quantitate the effects of *ras* p21 microinjection on individual components of the phosphoinositide pathway. Within 20 minutes of microinjection, levels of phosphatidylinositol 4,5-bisphosphate, inositol 1-phosphate, and inositol bisphosphate increased 1.5- to 2-fold. The most striking effects were on diacylglycerol, which increased 5-fold under the same conditions. In contrast, the normal *ras* p21 protein induced no detectable alteration in any of the metabolites analyzed. The earliest effects of the transforming p21 on phosphoinositol turnover were observable within 2 minutes, implying a very rapid effect of *ras* p21 on the enzymes involved in phospholipid metabolism.

GENES INVOLVED IN THE TRANSduction of signals required for normal cell proliferation commonly appear to be subverted in the neoplastic process (*1*). One such group is the highly conserved *ras* gene family. The p21 proteins encoded by *ras* genes bind guanine nucleotide (*2*) and have intrinsic guanosine 5′-triphosphatase (GTPase) activity (*3, 4*). They also appear to require attachment to the inner cell membrane for their function (*5*). In human tumors, *ras* oncogenes frequently have been found to be activated by point mutations at one of two major sites in their coding sequences (*6*). These activating lesions affect biochemical activities of the p21 protein, leading, for example, to impaired GTPase activity (*6*) or altered nucleotide exchange (*7*). Microinjection of bacterially expressed, mammalian transforming *ras* p21 proteins into NIH 3T3 cells leads to stimulation of DNA synthesis (*4, 8*). Thus, it has been suggested that by analogy with known G proteins, whose active state is the guanosine 5′-triphosphate (GTP)–bound but not guanosine 5′-diphosphate–bound form (*9*), *ras* p21 proteins may be regulatory proteins involved in transduction of signals that lead to DNA synthesis.

One major pathway involved in DNA synthesis induced by growth factors is mediated by phosphoinositide turnover according to the following scheme:



where PtdIns is phosphatidylinositol, PtdInsP is phosphatidylinositol phosphate, $PtdInsP_2$ is phosphatidylinositol 4,5-bisphosphate, and $InsP_3$ is inositol trisphosphate. Cleavage of phosphoinositides by phospholipase C (PLC) produces 1,2-diacylglycerol (DAG) and inositol phosphates (InsPs) (*10*). Whereas DAG acts as an essential cofactor for protein kinase C (PKC), inositol (1,4,5)trisphosphate, mobilizes Ca^{2+} from nonmitochondrial intracellular stores. Recent studies have indicated that cells chronically transformed by *ras* oncogenes exhibit increased phosphoinositide turnover (*11*). Moreover, such transformants have been reported to exhibit increased levels of DAG associated with a partial activation and downregulation of PKC (*11*). These findings have suggested that *ras* p21 proteins may directly or indirectly stimulate PLC-catalyzed breakdown of $PtdInsP_2$ or PtdIns. Because such investi-

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