This thesis is organized in two parts. The research in Part I was done with Dr. Michael D. Uhler. After Dr. Uhler left the Oregon Health Sciences University, the research in Part II was done with Dr. Gail M. Clinton. The two research projects should be taken independently of each other.

STRUCTURE, FUNCTION AND EXPRESSION OF TWO PROTEIN KINASES

by

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A DISSERTATION

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ABBREVIATIONS

Part I

cAMPdPK	cAMP dependent protein kinase
C	Catalytic subunit of cAMPdPK
CAP	Catabolite activator protein
CPT-cAMP	8-chlorophenylthio-cAMP
CRE	cAMP responsive element
PKI	Protein kinase inhibitor
R	Regulatory subunit of cAMPdPK

Part II

α	Antipeptide antibody
EGFR	Epidermal growth factor receptor
IR	Insulin receptor
PDGFR	Platelet derived growth factor receptor

Synthetic peptide abbreviations: ALE, DFG, HRD, P1, P3, SDV

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ABSTRACT

The research in Part I is an investigation of whether the cAMP induction of alkaline phosphatase in mouse L929 cells is mediated by activation of the cAMP dependent protein kinase. The L cell alkaline phosphatase cDNA was cloned in order to generate riboprobe specific for alkaline phosphatase. cAMP treatment of L cells in culture showed a correlation between changes in kinase activity, alkaline phosphatase activity, alkaline phosphatase mRNA levels and rate of alkaline phosphatase gene transcription. L cells were transfected with a Zn-inducible expression vector coding for the Cα isoform of cAMP dependent protein kinase so that the kinase activity in the transfected cells could be manipulated in the absence of cAMP. Zn treatment of clone Cα2 produced an increase in kinase activity, alkaline phosphatase enzyme activity and alkaline phosphatase mRNA, indicating that increased kinase activity is sufficient to induce expression of alkaline phosphatase in L cells. However Zn treatment of clone CαK72M, which was transfected with a mutant Cα expression vector, produced no increase in kinase activity and no alkaline phosphatase induction, suggesting that kinase activity is required to induce alkaline phosphatase.

The research in Part II is an examination of the functional importance of four highly conserved amino acid sequences in the kinase domain of the EGF receptor. For comparative purposes, and because they have been implicated in regulation of kinase activity, two autophosphorylation sites were also examined. Antipeptide antibodies were used for these studies because they are site-specific in their binding and therefore allow discrimination between these six selected EGF receptor sequences. Immunoprecipitation of the native protein by an antibody indicates that the sequence to which that particular

antibody binds is exposed on the surface of the protein, and inhibition of receptor autophosphorylation following antibody binding indicates that the cognizant EGF receptor sequence is important for catalysis. Two of the four kinase domain sequences (HRD and DFG) were found to be involved in catalysis and one of these (HRD) appears to be the least accessible of the six sequences examined. This accessibility could be important in regulation of kinase activity. The two autophosphorylation sites were exposed in the native protein and autophosphorylation had no effect on this exposure, which is in sharp contrast to what has been observed with the insulin receptor, another member of the growth factor receptor tyrosine kinase family of proteins.

Introduction

cAMP is a second messenger in both prokaryotes and eukaryotes. In prokaryotic cells, the protein which binds this second messenger and mediates all cAMP effects is CAP (catabolite activator protein), also called CRP (cAMP receptor protein) (2, 4). CAP is a dimeric protein whose amino-terminal domain binds two cAMP molecules in a β-roll structure. When cAMP is bound to CAP, the carboxy-terminal domain of the protein can bind to bacterial promoter sequences containing the consensus TGTGA and this binding enhances transcription of the gene. Because the genes activated by the cAMP-CAP complex usually code for enzymes involved in transport and utilization of sugars (e.g. lactose, arabinose, maltose), cAMP is a "hunger" signal in bacteria. When glucose levels are low, the cAMP concentration is high. This same inverse relationship between glucose and cAMP is found in eukaryotes, but in eukaryotic cells, the primary binding protein of cAMP is R, the regulatory subunit of cAMP dependent protein kinase (cAMPdPK) (2, 17, 19). Binding of two cAMP to each R subunit of the kinase holoenzyme dissociates the inactive tetramer, releasing free catalytic subunit .

$$R_2C_2 + 4\ cAMP\ \text{- - - - >}\ 2C + R_2(cAMP)_4$$

Because cAMP binding to the inactive holoenzyme dissociates the catalytic subunit, which is then free to phosphorylate cellular substrate proteins, it is the cAMPdPK which is thought to transduce the cAMP signal in eukaryotic cells.

cAMPdPK is a cytosolic serine/threonine protein kinase. It has been separated into two classes (type I and type II) by DEAE chromatography with type I eluting at a lower salt concentration than type II (6, 7, 17, 25). Studies of various bovine tissues have now revealed another form of type II kinase, so that bovine skeletal muscle represents predominantly type I, bovine heart is type II and bovine brain is a neural form of type II (2, 20). The two classes of cAMPdPK differ in molecular weight (I is 172 kD, II is 188 kD), in isoelectric point (pI of type I > pI of type II) and in the effect of increased salt concentrations on the dissociation of the holoenzyme (salt enhances dissociation of type II)

(2, 7). But in both classes, cAMP is freed from R after dissociation of the holoenzyme and the free R and C subunits reassociate in less than 2 minutes. This rapid reassociation means that kinase activity requires the continued presence of cAMP in the cytosol. Because phosphodiesterases in the cell act to decrease the concentration of cAMP, phosphodiesterase inhibitors such as IBMX or theophylline can be added to the media of cells grown in culture in order to reduce the level of exogenous cAMP analogue required to activate the kinase of these cells (6).

The differences between the two classes of cAMPdPK are primarily due to differences among the regulatory subunits making up the holoenzyme (19). These differences include: RI has a molecular weight of 49 kD, RII in most tissues is 54 kD and RII from brain is 51 kD; RII can be autophosphorylated by cAMPdPK and this inhibits reassociation with the C subunit, RI does not undergo autophosphorylation; RII is the more anionic regulatory subunit (2, 21, 25). But despite these difference, RI and RII display an overall similarity in structure and function (17). The N-terminal domain of the subunit is involved in R - R dimer formation while the carboxy terminus contains two tandem cAMP binding sites. The affinity of these two sites for cAMP differs and they can be experimentally differentiated by their preference in binding of synthetic cAMP analogues. The N-terminal domain of the regulatory subunit is separated from the C-terminal cAMP binding sites by an acidic hinge region and it is this region which binds R to the catalytic subunit in the holoenzyme. Within this region is a cAMPdPK substrate recognition sequence (R - R - X - S) which, in the case of RI, is a pseudosubstrate (R - R - G - A) while in RII it is a substrate sequence (R - R - V - S) (19). This substrate sequence probably occupies the peptide binding site of C in the holoenzyme and thereby acts as a competitive inhibitor of substrate. When cAMP binds to R, a structural change occurs which dissociates the holoenzyme, thereby freeing the catalytic subunit. Because binding of cAMP to R displays positive cooperativity, activation of the kinase is more sensitive to changes in cAMP concentration than would otherwise be true (2).

The catalytic subunit of cAMPdPK has now been characterized in terms of physical properties, composition and kinetic parameters. It is a 40 kD basic protein (10, 20, 21) whose amino-terminal glycine is myristilated (2, 4, 14), the Km for ATP is about 7uM (18) and it contains three cysteine residues of which one appears to be important for catalysis because it is blocked from iodoacetamide binding in the presence of Mg-ATP (14, 18). The separation of electrophoretically homogeneous C subunit protein into two distinct proteins by carboxymethyl cellulose chromatography (10) and the separation by nondenaturing gel electrophoresis of two distinct bands from a complex of C subunit bound to purified bovine protein kinase inhibitor (PKI) (24) were early indications that there might be two distinct C subunits of cAMPdPK. Two genetically distinct isoforms, Cα and Cβ, have now been cloned and characterized (16, 20, 21). These isoforms differ in mRNA size (Cα is 2.4 kb, Cβ is 4.3 kb), in isoelectric point , in dependence on Mg-ATP for binding to PKI and in their susceptibility to degradation by a protease specific for the C subunit (2, 10, 18, 21, 23). Cα is the more abundant isoform in most tissues, whether RI or RII is the dominant regulatory subunit (16, 21).

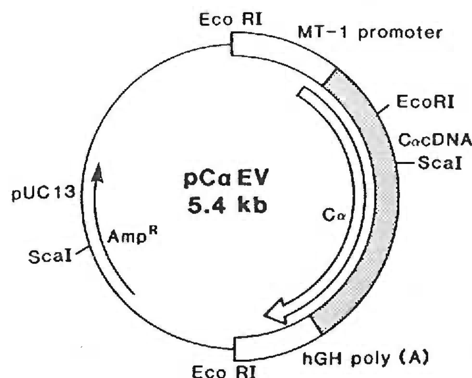
Protein phosphorylation is an essential regulatory mechanism in eukaryotic cells, a finely-tuned interplay of cellular protein kinases and protein phosphatases (2). The process by which an individual kinase is regulated may be unique to that kinase, but regulation is found in all kinases. Regulation of the cAMPdPK takes several forms, the most obvious of which is the presence of a distinct regulatory subunit whose high affinity binding (about .2nM) to the catalytic subunit inactivates the kinase (7). When the catalytic subunit is free, it is substrate to a specific protease which rapidly degrades it (1). In addition, the free C subunit may be bound by a heat- and acid-stable inhibitor protein (PKI) whose affinity for the peptide binding site of the kinase is greater than that of a kinase substrate, making it an extremely effective competitive inhibitor (24). Finally, there appears to be coordinate regulation of abundance of R and C subunits (2). Examination of mRNA levels for RI and C in various mouse tissues showed the abundance of RI to be equal to or greater than that

of C (20). The half-life of R in S49 lymphoma kin⁻ cells, a cell line which has reduced levels of C subunit, is shorter than the half-life in normal S49 cells, suggesting that the turnover of R in vivo is dependent upon how much C subunit is present (6, 17, 22). Both R and C are more rapidly degraded when they are not bound in the inactive kinase holoenzyme.

One of the cellular responses to cAMP is transcription of a specific gene, but the mechanism by which cAMP induces gene expression in eukaryotes is unknown. The sequence homology between bacterial CAP and the R subunit of eukaryotic cAMPdPK is so high that the cAMP binding sites of R have been made to fit into the crystallographic coordinates of CAP with only minor insertions and deletions (19). This sequence conservation has led to speculation that R, like CAP, modulates transcription by interacting with a specific nucleotide sequence of the regulated gene (13). In fact, examination of genes which are known to be responsive to cAMP (proenkephalin, phosphoenolpyruvate carboxykinase and prolactin) reveals conservation of specific sequences in the 5' flanking region of the genes (3). But although these cAMP-responsive elements (CRE) are conserved, it appears that the R subunit of cAMPdPK does not retain the DNA binding domain of bacterial CAP (2, 19). Attention has therefore shifted to examination of whether the cAMPdPK modulates increased nuclear transcription as it mediates other cellular responses to cAMP. Chromosomal proteins are known to affect the structure of genetic material and, thereby, the interaction of RNA polymerases with DNA (9). These nuclear proteins could be subject to regulation by phosphorylation just as cytoplasmic proteins are. Histone H1 is already known to be a substrate of cAMPdPK (2), as is RNA polymerase II (9). Both types of cAMPdPK have been found in the nuclei of mouse L cells and these nuclear kinases exhibit the same pattern of elution from a DEAE column and the same inhibition by PKI as that observed in cytoplasmic kinases (9). Fluorosceine isothiocyanate conjugated to bovine brain PKI has been used to identify a nuclear pool of free C subunit in adrenocortical tumor (Y-1) cells (12) and monospecific antisera have shown the presence of

C, RI and RII subunits in the nuclei of rat parotid gland cells (15). Somatostatin is a gene which exhibits a conserved CRE and conjugation of this 30-nucleotide CRE to an affinity column has allowed the isolation of a 43 kD protein which is phosphorylated upon incubation with the C subunit of cAMPdPK (11). These observations of nuclear cAMPdPK and the isolation of nuclear binding proteins which are substrates of the kinase has led to speculation that the cAMPdPK transduces the cAMP induction of gene expression through phosphorylation of a DNA-binding protein or through phosphorylation of a protein which interacts with other proteins involved in transcription.

If cAMPdPK mediates the cAMP induction of gene expression, a direct correlation between kinase activity and gene transcription should be observed. The work reported in Manuscript #1 is an investigation of whether such a correlation is seen in the cAMP induction of alkaline phosphatase in mouse L929 cells. Alkaline phosphatase is a membrane-bound glycoprotein (8) whose expression in L cells has been reported to increase 2000-fold when the cells were treated in culture for 7 days with dibutyryl cAMP (5). Based on in vitro translation of total RNA and immunoprecipitation of protein products, this induction of alkaline phosphatase was attributed to increased alkaline phosphatase mRNA levels. In order to determine whether cAMPdPK is directly involved in this induction, L cells were transfected with a Zn-inducible expression vector pCαEV which codes for the Cα isoform of cAMPdPK (22). The metallothionein promoter of this



expression vector allows efficient induction of Cα subunit expression, resulting in increased kinase activity in transfected cells. The isolation of a Zn-inductible L cell clone (Cα2) stably transfected with pCαEV allowed us to stimulate kinase activity in L cells in the absence of cAMP, thereby isolating kinase effects on induction of alkaline phosphatase. In order to attain the goal of monitoring alkaline phosphatase transcription and mRNA levels, we cloned the mouse alkaline phosphatase cDNA and ligated an Eco-RI fragment of this cDNA into pGEM-4 to generate riboprobe specific for alkaline phosphatase. We then used these tools to investigate whether a direct correlation exists in L929 cells between induction of alkaline phosphatase gene expression and cAMPdPK activity.

INDUCTION OF ALKALINE PHOSPHATASE IN MOUSE L CELLS BY OVEREXPRESSION OF THE CATALYTIC SUBUNIT OF cAMP DEPENDENT PROTEIN KINASE *

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Running Title: Induction of Alkaline Phosphatase by C Subunit

SUMMARY

Mouse L929 cells were used to study the mechanism of cAMP induction of alkaline phosphatase activity. Alkaline phosphatase activity in L cells was observed to increase 80-fold following 24 hours of treatment with 200 μM 8-chlorophenylthio-cAMP (CPT-cAMP) and 1000-fold following 7 days of treatment. The CPT-cAMP dose response of the alkaline phosphatase enzyme activity correlated well with cAMP-dependent protein kinase activation. A cDNA clone for the alkaline phosphatase was isolated and used to demonstrate a 7-fold increase in alkaline phosphatase mRNA levels after treatment of L cells with CPT-cAMP for 24 hours. A 6-fold increase in transcription of the alkaline phosphatase gene after CPT-cAMP treatment was demonstrated by *in vitro* nuclear transcription assay. In order to determine if cAMP-dependent protein kinase activity was mediating the transcriptional induction of alkaline phosphatase, L cells were transfected with expression vectors containing the metallothionein promoter and coding for the Cα isoform of the catalytic (C) subunit of cAMP-dependent protein kinase or a catalytic subunit in which lysine 72 had been mutated to methionine. Zinc treatment of cells containing the wild type C subunit expression vector produced an increase in protein kinase activity and an increase in alkaline phosphatase enzyme activity. Zinc treatment of cells containing the mutant C subunit expression vector produced an increase in the amount of a protein which was recognized by C subunit antibodies on Western blots, but these cells showed no increase in protein kinase or alkaline phosphatase activity. We conclude that the C subunit is sufficient for transcriptional induction of the alkaline phosphatase gene and that kinase activity is required for this induction.

Although the mechanism of transcriptional regulation by cAMP is well characterized in prokaryotes, it is poorly understood in eukaryotic organisms (Roesler *et al.*, 1988). In prokaryotes cAMP binds to catabolite activator protein (CAP)[1] which is itself a DNA binding protein (Weber *et al.*, 1982) . While cAMP is bound to CAP, it enhances transcriptional initiation by interacting with specific promoter sequences and RNA polymerase. In eukaryotes, however, the major receptor for cAMP is the regulatory (R) subunit of cAMP-dependent protein kinase (Beebe and Corbin, 1986; Taylor *et al.*, 1988). cAMP-dependent protein kinase exists as a catalytically inactive tetramer of two regulatory (R) subunits and two catalytic (C) subunits. Multiple isoforms of both the R and C subunits have been characterized but their functional significance is currently uncertain (Beebe and Corbin, 1986; Chrivia *et al.*, 1988). The binding of two molecules of cAMP to each R subunit results in the dissociation of the holoenzyme and release of catalytically active C subunit. The release of C subunit from the holoenzyme results in phosphorylation of serine and threonine residues in many cellular proteins. The majority of evidence to date would support a model for cAMP regulation of gene transcription where the C subunit released from the inactive holoenzyme is translocated to the nucleus (Boney *et al.*, 1983; Riabowol *et al.*, 1988). There the C subunit may phosphorylate proteins important in the regulation of gene transcription (Montminy and Bilezikjian, 1987; Grove *et al.*, 1987). It is unclear whether these phosphorylated proteins are themselves DNA binding proteins or whether they interact with other proteins which bind DNA.

However, other models for transcriptional regulation by cAMP have been proposed in the past. For example, the type II regulatory (RII) subunit has been proposed to have DNA binding properties (Nagamine and Reich, 1985; Shabb and Miller, 1986) and to have phosphorylation dependent topoisomerase activity (Constantinou *et al.*, 1985). Since the R subunits have also been reported to be translocated to the nucleus after elevation of cAMP levels (Mednieks and Jungmann, 1982; Schwartz and Costa, 1980; Nesterova *et al.*,

1981), it has been proposed that transcriptional regulation may not involve phosphorylation by the C subunit but rather some property of the R subunit (Nagamine and Reich, 1985).

The expression of many eukaryotic genes has been reported to be transcriptionally induced by cAMP, but one of the most dramatic inductions reported to date has been the induction of alkaline phosphatase activity in mouse L cells (Firestone and Heath, 1981). Alkaline phosphatase is a membrane bound glycoprotein which exists in several isoforms that can be experimentally distinguished by differences in antigenicity and inhibitor sensitivity (Gum and Raetz, 1985). Bt_2cAMP treatment of mouse L cells resulted in a 2000-fold increase in the specific activity of alkaline phosphatase in cell extracts. Furthermore, *in vitro* translation of mRNA from control and cAMP treated cells suggested that this induction was due to an increased abundance of the mRNA coding for alkaline phosphatase (Firestone and Heath, 1981). Although the mechanism of induction of enzyme activity was not clearly demonstrated, this system offers several advantages for the analysis of cAMP-dependent protein kinase function in the regulation of gene expression. First, L cells are easily transfected with a high efficiency and expression vectors are available for subunits of cAMP-dependent protein kinase. Secondly, the alkaline phosphatase activity is easily determined using a number of substrates in both cell extracts and in a screening assay for analysis of L cell colonies (Gum and Raetz, 1983). Finally, the low level of basal expression and the magnitude of induction allows sensitive detection of effects on gene transcription.

The availability of expression vectors which allow the overproduction of the C subunit of cAMP-dependent protein kinase (Uhler and McKnight, 1987) enabled us to test whether the C subunit has a direct effect on gene transcription. In this paper we present evidence that the induction of the alkaline phosphatase activity occurs at a transcriptional level. We also present evidence that overexpression of the C subunit is sufficient to induce alkaline phosphatase enzyme activity and mRNA levels. Finally, we report that a mutant C subunit in which a lysine at position 72 has been altered to methionine by *in vitro*

mutagenesis is catalytically inactive and unable to induce alkaline phosphatase activity when overexpressed in mouse L cells.

MATERIALS AND METHODS

Cell culture and transfection of mouse L cells: NCTC clone 929 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium containing 10% horse serum (DMEM/HS). Addition of $ZnSO_4$ (Sigma, tissue culture grade) from a 10 mM stock solution or CPT-cAMP (Sigma) as a 3 mM stock solution in DMEM to the L cell cultures was preceded by changing the media to fresh DMEM containing 10% fetal calf serum (DMEM/FCS) 4 h before treatment . For transfection, confluent mouse L cells were split 1:20 the day before transfection. A 10 cm plate of cells was co-transfected with pKOneo and the appropriate C subunit expression vector as described (Uhler and McKnight, 1987). G-418 resistant colonies were selected in DMEM/HS containing 750 μg/ml G-418. The resulting G-418 resistant clones were screened for Zn-inducible expression by culturing cells in DMEM/HS containing 200 μM $ZnSO_4$ for 16 h and assaying for induction of Cα mRNA levels using a SP6 solution hybridization assay (McKnight *et al.*, 1988).

Expression vector construction and mutagenesis: Cα2 cells were generated by transfection of L cells with an expression vector identical to pCαEV except that the *BamHI/SacI* fragment containing the 5' region of the mouse metallothionein promoter (Glanville et al., 1981) was replaced with the BamHI/Sac fragment of the MMTV promoter (Majors and Varmus, 1983). This expression vector was constructed in an attempt to generate a promoter that would respond to both Zn and glucocorticoids. However the induction by Zn was unaffected by the change in promoter structure and the glucocorticoid response was only 3-fold over control cells. The Cα protein coding region is identical in this vector and the previously described pCαEV (Uhler and McKnight, 1987). The pCαK72M expression vector was constructed by oligonucleotide mutagenesis (Zoller and Smith, 1984) using an oligonucleotide with the sequence

GTCGAAGATCATCATGGCGTA to mutagenize the sense strand of the *Sac*I/*Pvu*I fragment of pCαEV in M13mp18. The entire *Sac*I/*Bgl*II fragment was sequenced following mutagenesis and subcloned into pCαEV to generate pCαK72M which is identical to pCαEV except at nucleotide 390 of the published Cα cDNA sequence where the codon AAG coding for lysine 72 has been changed to ATG coding for methionine and at nucleotide 397 where the leucine codon has been changed from TTA to TTC to create a new *Taq*I recognition sequence. Hence, the only difference in the proteins produced by pCαEV and pCαK72M is that the latter protein has a methionine at position 72 while the former retains the lysine at this position seen in the wild-type Cα sequence (Uhler et al, 1986)

Alkaline phosphatase assay: For quantitation of alkaline phosphatase enzyme activity, the medium was aspirated from 10 cm culture plates and the plates were washed twice with cold TBS (.01 M Tris, 0.15 M NaCl/pH 7.3). The cells were scraped off into cold TBS and pelleted at 800 rpm for 5 minutes. The pellet was then resuspended in cold TBS and sonicated. This cell extract was assayed for alkaline phosphatase activity using p-nitrophenylphosphate as the substrate as described (Firestone and Heath, 1981). Activity is reported as units/gm total protein where 1 unit represents 1 μmole of p-nitrophenylphosphate hydrolyzed per minute. All protein concentrations were determined spectrophotometrically using a dye binding assay (Biorad).

Kinase assay: Cell pellets were resuspended in homogenization buffer (10 mM $NaPO_4$ (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose) and the protein concentration adjusted to 2 mg/ml. Kinase assays were performed as previously described (Uhler and McKnight, 1987). Endogenously active kinase was determined in the absence of any added cAMP in the kinase assay and total kinase activity was determined in the presence of 10 μM cAMP.

Cloning of the mouse L cell alkaline phosphatase cDNA. Twenty 15 cm plates of L cells were treated for 48 h with 1 mM CPT cAMP. Poly(A) RNA was isolated from these

plates using guanidinium isothiocyanate for isolation of total RNA and oligo(dT)cellulose for poly(A) RNA purification as described (Uhler *et al.*, 1986b). A cDNA library of 400,000 independent clones in λgt10 was constructed as described previously (Uhler *et al.*, 1986b) and screened using an oligonucleotide (ATGATCTCACCATTTTTAGTACTGGCCATCGGCACCTGCCTTACCAAC) corresponding to the first 48 coding nucleotides of the published mouse placental cDNA (Terao and Mintz, 1987). Two positively hybridizing plaques were identified and purified. Both phage contained cDNA inserts 2.5 kb in length and the cDNA insert from one of these phage was then subcloned into M13mp18 and partially sequenced by dideoxy sequencing (Messing, 1983).

<u>SP6 quantitation of messenger RNA</u>: The cDNA fragments were subcloned into pGEM-4 (Promega Biotec) and ^{35}S-labeled antisense transcripts for both the alkaline phosphatase cDNA and the Cα subunit of cAMP-dependent protein kinase were generated using SP6 polymerase as described (McKnight *et al.*, 1988). The radiolabeled antisense transcripts were used for detection of mRNA by hybridization and M13mp18 constructs containing the sense strand were used as standards for mRNA quantitation in a solution hybridization assay (McKnight *et al.*, 1988)

<u>In vitro nuclear transcription:</u> Nuclei were isolated as described (McKnight and Palmiter, 1979) from three 10 cm plates of L cells for each of the following treatments: control, 1 h of 3 mM CPT-cAMP treatment, and 2 h of 3 mM CPT-cAMP treatment. Nuclei (about 50 µg of DNA equivalent) were incubated for 1 hour at 26°C with 300 µCi of ^{32}P-UTP (800 Ci/mmol, New England Nuclear) (McKnight and Palmiter, 1979). ^{32}P-labeled RNA was isolated and hybridized to nitrocellulose filters containing 10 µg of M13mp18 containing the 2.5 kb alkaline phosphatase cDNA in the sense orientation for determination of background hybridization, or M13mp18 containing the 2.5 kb alkaline phosphatase cDNA in the antisense orientation for determination of the alkaline phosphatase gene transcription rate, or M13mp18 containing the 2.2 kb Cα subunit cDNA in the antisense orientation for

determination of the Cα subunit gene transcription rate. A ^{3}H-labeled cRNA for the Cα subunit was used to determine the efficiency of hybridization, which was 33±3% for all hybridizations.

Western blot analysis: Cell extracts prepared as for the kinase assay were boiled for 5 minutes after adding sample buffer (Uhler and McKnight, 1987). The denatured samples were cooled on ice, electrophoresed on a 10% SDS-PAGE gel (Laemmli, 1970) and transferred to nitrocellulose. Incubation of the blot with either the R or C subunit antibody was followed by incubation with alkaline phosphatase coupled anti-rabbit IgG (Promega Biotec) to visualize the bands. C subunit antibody was the generous gift of Dr. Brian Hemmings (Friedrich Miescher-Institut, Basel) and type I regulatory (RI) subunit antibody was kindly provided by Dr. Stan McKnight (University of Washington, Seattle).

RNA isolation and Northern blot analysis: RNA was isolated as described above for alkaline phosphatase cDNA preparation. RNA samples and RNA size standards (Bethesda Research Labs) were loaded onto a formaldehyde 1% agarose gel for Northern analysis as previously described (Uhler *et al.*, 1986b).

RESULTS

As had been previously reported (Firestone and Heath, 1981; Gum and Raetz, 1985), we observed a dramatic increase in L cell alkaline phosphatase enzyme activity after treatment with the cAMP analogue CPT-cAMP (Fig 1). This cAMP analogue was chosen for these studies because it has previously been shown to be more resistant to degradation by phosphodiesterases and is a more potent activator of cAMP-dependent protein kinase than other cAMP analogues such as dibutyryl cAMP and 8-bromo cAMP (Miller *et al.*, 1975). In addition, its metabolism does not generate butyrate which has been shown in some systems to induce alkaline phosphatase (Gum *et al.*, 1987). The first increase in enzyme activity was detected between 6 and 8 h after treatment. An 80-fold increase was observed at 24 h after CPT-cAMP treatment with an increase from 0.14 U/gm total cellular protein to 10.6 U/gm. After 7 days of treatment the response was saturated at 50 U/gm protein resulting in a 1000-fold increase over untreated L cells.

To assess the possible correlation between alkaline phosphatase induction and cAMP-dependent protein kinase activation, dose response curves for both alkaline phosphatase activity and cAMP-dependent protein kinase activity ratio were generated after 24 h of treatment with various concentrations of CPT-cAMP. This experiment was performed at 24 h of treatment when alkaline phosphatase activity was still linearly increasing, even at maximal CPT-cAMP concentration. As shown in Fig. 2, there is a significant correlation between the degree of kinase activation and the induction of alkaline phosphatase enzyme activity in mouse L cells at higher levels of CPT-cAMP (> 300 μM CPT-cAMP). At lower concentrations, the basal level of kinase activity (15-18% activation) was not affected by CPT-cAMP treatment. This high level of basal activation may be due to the actual activity ratio in the cells or due to activation during preparation of cell extracts for assay of kinase activity. Neither kinase activation nor alkaline phosphatase induction was saturated at 3 mM CPT-cAMP. Higher concentrations of CPT-cAMP were

difficult to obtain due to the low solubility of CPT-cAMP in DMEM. The maximum activity ratio observed was 0.60, indicating that after 24 h of treatment with 3mM CPT-cAMP 60% of the cAMP-dependent protein kinase had been activated. It was also noted that during this time a 73% reduction in the total amount of protein kinase activity was seen upon comparing the total kinase activity in control L cells (680 U/mg) to that of cells treated for 24 h with 3 mM CPT-cAMP (250 U/mg).

In order to determine if the increase in alkaline phosphatase enzyme activity was due to an increase in the amount of mRNA coding for alkaline phosphatase, an alkaline phosphatase cDNA clone was isolated. Using an oligonucleotide probe based on the published mouse placental alkaline phosphatase cDNA sequence (Terao and Mintz, 1987) we screened a cDNA library from CPT-cAMP treated L cells and isolated an alkaline phosphatase cDNA. Sequencing of approximately 200 bp at both the 5' end (corresponding to nucleotides 85 to 279 of the mouse placental cDNA sequence) and 3' end (corresponding to nucleotides 2252 to 2460) of this cDNA showed it to be 98% identical in these regions to the published placental cDNA. This cDNA was used to generate radiolabeled probe for Northern blot analysis of poly(A) RNA from control and CPT-cAMP treated mouse L cells. As shown in Fig. 3, the Northern blot indicates a substantial induction of the 2.5 kb alkaline phosphatase mRNA in the poly(A) RNA from CPT-cAMP treated cells compared to control cells. Densitometric scanning of the autoradiogram indicated at least a 10-fold increase in the amount of radiolabeled cDNA probe hybridizing to this 2.5 kb band after CPT-cAMP treatment. The 2.5 kb size of this mRNA correlates well with the alkaline phosphatase mRNA described and cloned from mouse placenta (Terao and Mintz, 1987). The same poly(A) RNA was used for quantitation of alkaline phosphatase mRNA by a solution hybridization assay and the results indicated the alkaline phosphatase mRNA increased at least 7-fold from a background basal level of .0002% to .0014% of the RNA after CPT-cAMP treatment. This estimate of mRNA abundance is in

agreement with the prevalence of alkaline phosphatase cDNA clones in the CPT-cAMP induced L cell library (2 clones out of 400,000 screened or .0005%).

The alkaline phosphatase cDNA was also used to determine whether this increase in mRNA was due to increased alkaline phosphatase gene transcription. Nuclei were isolated from control L cells and from L cells treated with 3 mM CPT-cAMP for 1 or 2 h and RNA transcripts labeled *in vitro* in the presence of ^{32}P-UTP. The labeled RNA was hybridized to M13 single stranded phage DNA containing either sense or antisense alkaline phosphatase cDNA which had been fixed to nitrocellulose filters. The sense alkaline phosphatase filters served to determine background hybridization. Cα subunit cDNA cloned in M13 was also fixed to nitrocellulose to determine the rate of Cα subunit gene transcription. As shown in Fig. 4, alkaline phosphatase gene transcription increased from 9 parts per million (ppm) in control L cells to 22 ppm after 1 hour of CPT-cAMP treatment and 54 ppm after 2 h of CPT-cAMP treatment. Cα subunit gene transcription in the same nuclei did not change with CPT-cAMP treatment and remained constant at approximately 13 ppm.

To determine directly if cAMP-dependent protein kinase was involved in the transcriptional response of the alkaline phosphatase gene to cAMP, L cells were co-transfected with an inducible expression vector for the Cα subunit of cAMP-dependent protein kinase and an expression vector for the selectable marker, neomycin phosphotransferase. The expression vector for the Cα subunit of cAMP-dependent protein kinase has previously been demonstrated to code for a catalytically active protein in mouse NIH/3T3 cells (Uhler and McKnight, 1987). G-418 resistant clones were isolated and characterized for their expression of the Cα subunit mRNA in the presence and absence of Zn. L cell clones expressing a mutated form of the Cα subunit in which the lysine at position 72 was changed to methionine were also isolated by transfection with a second expression vector. This lysine residue has been demonstated by affinity labeling experiments to be involved in ATP binding by the catalytic subunit (Zoller *et al.*, 1979). In

vitro mutations which change the analogous lysine to methionine in other protein kinases have been shown to inactivate those protein kinases (Chou *et al.*, 1987; Weinmaster *et al.*, 1986; Snyder *et al.*, 1985; Kamps and Sefton, 1986). In both L cell transfections, clones which showed induced Cα subunit mRNA in response to Zn treatment were characterized with respect to induction of kinase activity and alkaline phosphatase activity.

Fig. 5 shows the induction of alkaline phosphatase activity when a clone (designated Cα2) containing the wild type Cα subunit expression vector is treated with 200 μM $ZnSO_4$ in culture. Alkaline phosphatase activity increases 13-fold from 0.15 U/gm to 2.0 U/gm over the course of 48 h. Poly(A) RNA from the Cα2 cells was isolated after no treatment or after Zn or CPT-cAMP treatment and subjected to Northern blot analysis using the alkaline phosphatase cDNA as radiolabeled probe. As is shown in Fig. 6, Zn treatment increased the level of alkaline phosphatase mRNA in the clone although not as well as cAMP treatment.

A more detailed study of Zn induction of the expression vectors in the transfected L cells was performed by comparing the effect of Zn on wild type L cells, the Cα2 clone containing the Cα subunit expression vector, and the CαK72M13 clone containing the mutant Cα subunit expression vector in which the lysine at position 72 has been changed to methionine. Fig. 7 shows the level of Cα subunit mRNA in these three different cell types in the absence or presence of $ZnSO_4$. Wild type L cells contain approximately 50 copies of Cα mRNA per cell and Zn treatment has no effect on the level of Cα mRNA in these cells. The Cα2 cells, which have been transfected with the Cα expression vector, contain approximately 80 copies of Cα mRNA per cell in the absence of Zn but this increases to 400 copies per cell after Zn treatment. In the CαK72M13 clone, harboring the mutant Cα subunit expression vector, the Cα subunit mRNA level increases from 500 molecules per cell under basal conditions to 7000 molecules per cell after Zn treatment.

The effect of these Cα subunit mRNA levels on kinase activity in the three different cell types is shown in Fig. 8. There is no effect of Zn on kinase activity in wild type L

cells where endogenously active kinase activity is 50 units/mg protein and total kinase activity is 800 units/mg. Zn treatment of the Cα2 cell line however increases the endogenously active kinase activity from 80 units/mg to 240 units/mg. This same treatment increases the total kinase from 900 units/mg in the basal state to 2100 units/mg after Zn induction of transcription from the Cα expression vector. In the CαK72M13 cells, however, the endogenously active kinase activities are 50 units/mg protein in the absence or presence of Zn and total kinase activities are 450 units/mg protein independent of whether or not the cells have been treated with Zn. Thus, even though the CαK72M13 cells are expressing a 140-fold excess of the mutant Cα mRNA, they show no increase in kinase activity but rather a consistent decrease of 30 to 50 percent in total kinase activity was observed in these when compared to L cells. This would seem to indicate that the C subunit containing the lysine to methionine change at position 72 possesses little if any kinase activity.

Cell extracts from Cα2 and CαK72M18, a cell line expressing levels of mutant Cα subunit similar to those of the cell line CαK72M13, were subjected to Western blot analysis to study the protein produced by the expression vectors. Zn treatment of Cα2 cells produced an increased level of C subunit that comigrates on SDS-PAGE with the endogenous C subunit in L cells. There was also an increase in immunoreactivity of a band that comigrates with the RI subunit. This is consistent with a similar compensation that was described previously for mouse NIH 3T3 cells (Uhler and McKnight, 1987). Zn treatment of CαK72M13 or CαK72M18 cells however caused an increase in the amount of a protein immunologically related to the C subunit but which migrated slightly faster in SDS-PAGE than endogenous C subunit. Although small increases in RI subunit were occasionally observed after Zn treatment of CαK72M cells, the RI subunit compensation was not as large as that seen in Cα2 cells when the wild-type Cα subunit was overexpressed.

The ability of each of these L cell clones to induce alkaline phosphatase in response to Zn and CPT-cAMP was tested. As shown in Fig. 10, L cells do not show increased alkaline phosphatase in response to Zn treatment but did respond to CPT-cAMP treatment with a 40-fold increase in alkaline phosphatase activity from .11 U/gm in the control cells to 4.4 U/gm in the CPT-cAMP treated cells. In contrast to the L cells, the Cα2 cells responded to Zn treatment with a 20-fold increase in alkaline phosphatase activity from .06 U/gm to 1.2 U/gm. These same cells showed an 80-fold increase in alkaline phosphatase activity when cells treated with CPT-cAMP (5.0 U/gm) are compared with untreated cells (.06 U/gm). The Cα2K72M13 cells did not show an increase in alkaline phosphatase activity with Zn treatment (.28 U/gm in conrol cells and .16 U/gm in Zn treated cells), but did respond to CPT-cAMP with a 10-fold increase in alkaline phosphatase activity (.28 U/gm in control cells and 3.3 U/gm in CPT-cAMP treated cells). Cells expressing the CαK72M subunit did show induction of both Cα subunit mRNA and Cα subunit protein as shown in Figs. 7 and 9 respectively, but this mutant protein lacks kinase activity (Fig. 8). Furthermore, the mutant C subunit protein in the CαK72M13 cells appears to be incapable of inducing the alkaline phosphatase gene in response to Zn, although the endogeneous Cα subunit gene product is still able to induce the alkaline phosphatase gene in response to CPT-cAMP.

DISCUSSION

The results shown demonstrate that the previously reported induction of alkaline phosphatase by dibutyryl cAMP can also be mimicked by CPT-cAMP. This lends support to the notion that induction of alkaline phosphatase occurs through a cAMP-dependent mechanism and not through the effect of butyrate. The observation that induction of alkaline phosphatase enzyme activity correlates with activation of cAMP-dependent protein kinase suggested that effect of cAMP may be mediated by activation of the kinase. It is interesting to note that even at 3 mM CPT-cAMP the induction of alkaline phosphatase activity has not reached a maximum (Fig. 2). This is in contrast to many other cellular responses to cAMP which are maximally induced at concentrations of cAMP which activate a much smaller fraction of the total cellular cAMP-dependent protein kinase. For example, in perfused liver, phosphorylase is maximally stimulated by epinephrine concentrations which activate only 30 - 35% of the total cAMP-dependent protein kinase (Keely *et al.*, 1975). Maximal stimulation of ACTH release occurs at concentrations of corticotropin releasing factor (CRF) which activate only 50% of the type I kinase while type II kinase is not activated at all by CRF (Litvin *et al.*, 1984). It is possible that this difference in response to cAMP-dependent protein kinase activation between alkaline phosphatase induction and other cellular responses reflects differences in the kinase isoforms which mediate these various cellular cAMP responses or that these cellular functions occur in different cellular compartments.

The down-regulation of cAMP-dependent protein kinase seen here in mouse L cells (73% in 24 hr with 3 mM CPT-cAMP) has also been seen in porcine LLC-PK_1 cells. Treatment of these cells with either 8-bromo cAMP or forskolin together with isobutylmethylxanthine resulted in a similar loss of enzyme activity. This loss of activity

was shown to be due to specific proteolysis of the C subunit and represents one cellular mechanism for down-regulating the activated kinase (Hemmings, 1986).

The present finding that the cDNA for the alkaline phosphatase induced in mouse L cells is very similar or identical to the mouse placental alkaline phosphatase cDNA was not unexpected. Three isoforms of human alkaline phosphatase have been described: an intestinal isoform, a placental isoform, and a bone-liver-kidney (B/L/K) isoform. In humans it is the B/L/K isoform which is cAMP inducible. Genetic evidence suggests, however, that only two forms of alkaline phosphatase exist in the mouse (Terao *et al.*, 1988), a placental and an intestinal isoform. Furthermore, the placental cDNA was used to show that the placental isoform is expressed in tissues other than the placenta. Biochemical characterization of the potency of various peptide inhibitors has also suggested that in mouse the placental and L cell alkaline phosphatase are very similar (Gum and Raetz, 1983). Thus in mouse it appears as if the B/L/K and placental alkaline phosphatases are encoded by the same gene which is also the form expressed in L cells.

The previous finding that the amount of mRNA coding for alkaline phosphatase as determined by *in vitro* translation increases with Bt_2cAMP treatment (Firestone and Heath, 1981) is in complete agreement with present experiments which determined that alkaline phosphatase mRNA levels increased at least 7-fold and that this increase was due in part to an increase in alkaline phosphatase gene transcription. From the data presented it is not possible to determine whether this transcriptional induction can account for the total increase in alkaline phosphatase mRNA, since the duration of transcriptional induction has not been determined and the effect of cAMP on alkaline phosphatase mRNA is unknown at this time. It is also not clear from experiments presented here whether the cAMP induction of alkaline phosphatase gene transcription is a primary or secondary induction. Further experiments to characterize the sensitivity of the transcriptional induction to cycloheximide or characterization of the alkaline phosphatase promoter will be necessary to answer this

question. It is interesting to note however that the human B/L/K alkaline phosphatase promoter region does not contain a classic cAMP response element (Weiss *et al.*, 1988).

Since the induction of overexpression of the Cα subunit in Cα2 cells by Zn treatment was sufficient to induce alkaline phosphatase activity and alkaline phosphatase mRNA levels, cAMP and the R subunits of cAMP-dependent protein kinase do not appear to play any role other than to regulate the kinase activity of the C subunit. Several other Cα overexpressing cell lines were generated during the course of these experiments and Zn induction of Cα mRNA levels had similar effects on alkaline phosphatase activity, but the Cα2 cells were convenient for the studies present here because of their low basal level of Cα mRNA expression. Although overexpression of the Cα subunit does lead to increased levels of R subunit protein, this R subunit results from an increased stability of the R subunit in the holoenzyme complex as compared to the free R subunit (Steinberg and Agard, 1981) and is not due to an increase in the mRNA for the R subunit (Uhler and McKnight, 1987). In addition, the compensating R subunit protein is complexed in an inactive holoenzyme complex with C subunit as determined by kinase activity (Uhler and McKnight, 1987).

From the experimental data presented here, induction of alkaline phosphatase activity requires the kinase activity of the Cα subunit. Expression of a mutant Cα subunit in which the lysine residue at position 72 in the protein has been changed to methionine is incapable of inducing alkaline phosphatase activity. This lysine residue has been shown by affinity labeling to be involved in ATP binding to the C subunit (Zoller *et al.*, 1981). This lysine is part of a sequence motif strictly conserved among protein kinases and mutagenesis of this lysine residue has been shown to abolish kinase activity for other protein kinases including the human insulin receptor (Chou *et al.*, 1987), the *fps* oncogene (Weinmaster *et al.*, 1986), and *v-src* (Snyder *et al.*, 1985; Kamps and Sefton, 1986). The present finding that the analogous mutation in the Cα subunit abolishes kinase activity is therefore not surprising. However, the fact that this protein is unable to induce alkaline phosphatase

activity in intact cells suggests that protein phosphorylation by the Cα subunit plays a central role in cAMP regulation of alkaline phosphatase gene expression. Several differences were noted between the CαK72M mutant protein and the wild-type Cα protein. First, the CαK72M mutant protein consistently migrated more rapidly than the wild-type protein on SDS-PAGE. Secondly, the CαK72M protein appeared to be less stable than the wild type protein in that much higher levels of the CαK72M mRNA as compared to wild-type Cα mRNA were required to see similar amounts of the two proteins (see Figs. 7 and 9). Finally, the CαK72M mutant was not able to stabilize the RI subunit as well as the wild-type Cα subunit. Since the mutated lysine residue has been shown to play a role in ATP binding (Zoller *et al.*, 1979), it is possible that autophosphorylation of the C subunit has been affected and that lack of autophosphorylation may in turn affect conformation as seen by altered migration in SDS-PAGE, proteolytic degradation, and holoenzyme formation of the Cα subunit. Alternatively, it may be the simple change in charge from positively charged lysine to neutral methionine that alters the rate of migration of the mutant protein.

The conclusion that phosphorylation by the C subunit of cAMP-dependent protein kinase is required for transcriptional regulation of the alkaline phosphatase gene by cAMP is consistent with recently reported results in other systems. An expression vector for the protein kinase inhibitor peptide has been shown to inhibit the cAMP stimulation of transcription of the human enkephalin promoter in a transient expression assay system (Grove *et al.*, 1987). In addition, microinjection of the C subunit has been shown to stimulate transcription from the vasoactive intestinal peptide gene promoter and the *c-fos* promoter, whereas microinjection of the R subunits did not stimulate transcription from these promoters (Riabowol *et al.*, 1988). Although these studies clearly implicate the kinase activity of the C subunit in regulation of gene transcription, in neither of these cases was it possible to assay for cAMP-dependent protein kinase in the cell and directly correlate kinase activity with the transcriptional response. The mouse L cell alkaline phosphatase

system presented here has some clear advantages over other gene transcription systems and has facilitated the demonstration that the kinase activity of the C subunit of cAMP-dependent protein kinase is sufficient and necessary for transcriptional induction of the mouse L cell alkaline phosphatase gene.

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FOOTNOTES

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[1] The abbreviations used are: CPT-cAMP, 8-chlorophehylthio-cAMP; C, catalytic subunit; CAP, catabolite activator protein; R, regulatory subunit; Bt_2cAMP, dibutyryl cAMP; DMEM, Dulbecco's modified Eagle's meidum; CRF, corticotropin releasing factor; kb, kilobase(s); SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Conclusion

The data presented here demonstrate that cAMP treatment of L929 cells in culture resulted in increased alkaline phosphatase enzyme activity and higher levels of alkaline phosphatase mRNA as determined by Northern analysis. This cAMP induction of alkaline phosphatase is in agreement with an earlier study which used in vitro translation of total RNA and immunoprecipitation of protein products to demonstrate increased alkaline phosphatase mRNA levels (5). In addition, our data demonstrated an increase in cAMPdPK activity and alkaline phosphatase gene transcription in the cAMP-treated cells. Zn-induced overexpression of the Cα subunit in Cα2 cells produced a similar induction of alkaline phosphatase, suggesting that increased kinase activity was sufficient to mediate this increase in alkaline phosphatase enzyme activity and mRNA levels. In fact, kinase activity was required for the regulation of alkaline phosphatase gene expression because Zn-induced overexpression of a mutant Cα subunit (CαK72M18) which did not exhibit kinase activity was unable to induce alkaline phosphatase in L cells. This correlation of kinase activity with expression of alkaline phosphatase in L cells is in agreement with the proposals discussed above that cAMP induction of gene expression is mediated by the cAMPdPK.

Whether the induction of alkaline phosphatase by cAMP is solely due to increased alkaline phosphatase gene transcription cannot be determined by the data presented here. There was an increase in transcription rate, but it remains to be seen whether there was any change in alkaline phosphatase mRNA stability. In addition, we have not yet examined wherher the induction of alkaline phosphatase transcription is a primary or secondary effect.

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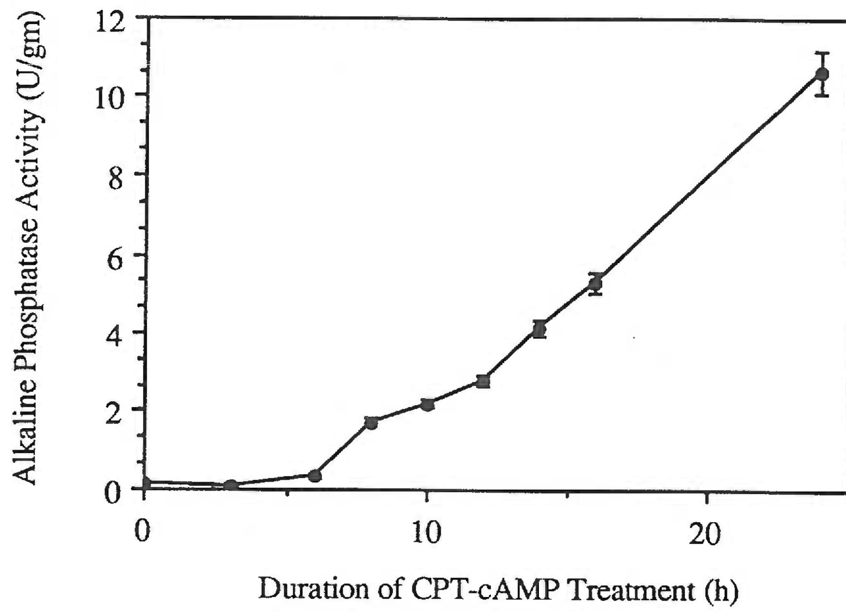
24. Whitehouse, S. and Walsh, D.A. (1982) Mg-ATP-dependent Interaction of the Inhibitor Protein of the cAMP Dependent Protein Kinase with the Catalytic Subunit JBC 258: 3682 - 3692.

25. Zoller, M.J., Kerlavage, A.R. and Taylor, S.S. (1979) Structural Comparisons of cAMP Dependent Protein Kinases I and II from Porcine Skeletal Muscle JBC 254: 2408 - 2412.

FIG. 1. **The effect of CPT-cAMP on mouse L cell alkaline phosphatase activity.** Confluent cultures of mouse L cells in 10 cm tissue culture dishes were incubated in DMEM containing 10% fetal calf serum and 3 mM CPT-cAMP. At the times indicated the cultures were harvested and lysates prepared and assayed for alkaline phosphatase activity and protein content. Each point represents the mean ± S. D. for triplicate determinations.



Alkaline Phosphatase Activity (U/gm)
12
10
8
6
4
2
0
0
10
20
Duration of CPT-cAMP Treatment (h)

FIG. 2. **The effect of CPT-cAMP on the activity ratio of cAMP-dependent protein kinase and alkaline phosphatase activity in mouse L cells.** Confluent cultures in 10 cm tissue culture dishes were incubated in DMEM containing 10% fetal calf serum and the indicated concentration of CPT-cAMP. After 24 h the cells were harvested and separated into two aliquots. One aliquot was used for determination of cAMP-dependent protein kinase activities in the presence or absence of 10 μM cAMP to determine endogenously active and total kinase activity respectively. The activity ratio shown (open squares) is the ratio of protein kinase activity determined in the absence of exogenous cAMP to the protein kinase activity determined in the presence of 10 μM cAMP. The other aliquot was used for determination of alkaline phosphatase activity (filled squares). Each value represents the mean ± S. D. for triplicate determinations.

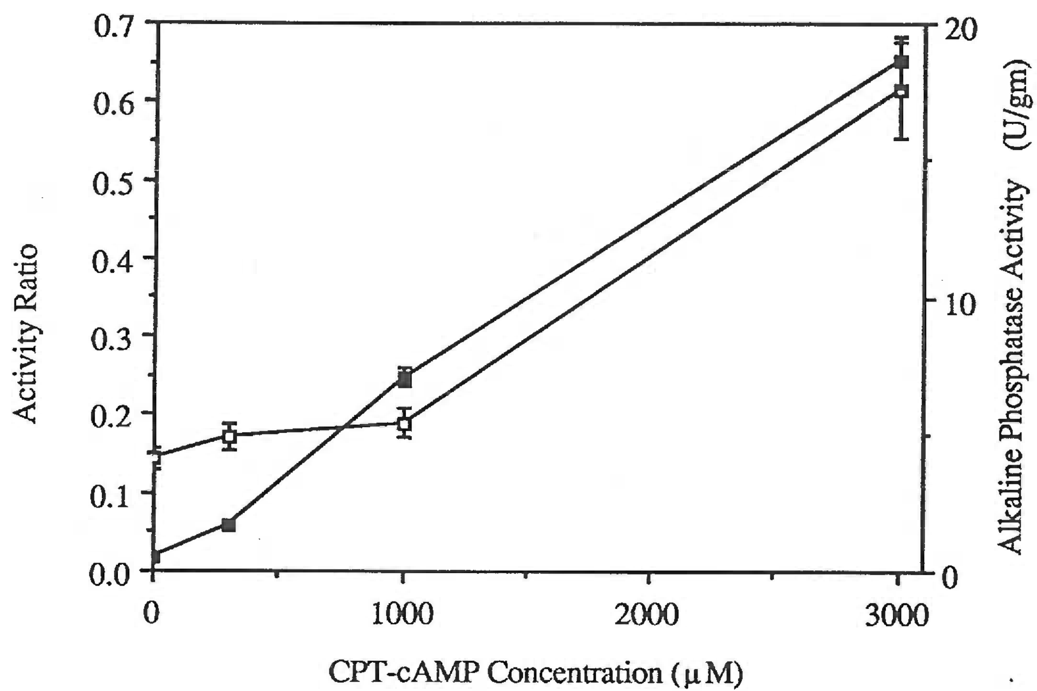

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Activity Ratio
20
10
0
Alkaline Phosphatase Activity (U/gm)
0
1000
2000
3000
CPT-cAMP Concentration (μM)

FIG. 3. **Northern blot analysis of RNA isolated from control and CPT-cAMP treated L cells.** Samples of poly(A) RNA from control and CPT-cAMP treated L cells were subjected to electrophoresis and hybridization analysis as described under "Materials and Methods". The 2.5 kb mouse L cell alkaline phosphatase cDNA was used to generate radiolabeled probe. The size of the hybridizing band in the cAMP treated lane was determined using RNA molecular weight markers and staining the gel before transfer to nitrocellulose.

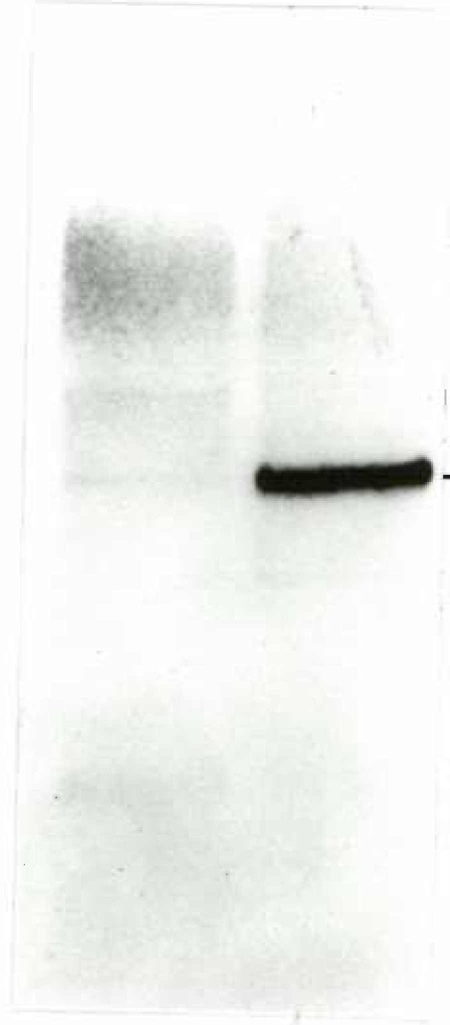

Control
cAMP
2.5 kb

FIG. 4. **Transcription rates for the alkaline phosphatase and Cα subunit genes in control and CPT-cAMP treated L cells.** Nuclei were prepared from confluent 10 cm tissue culture dishes which had been treated with DMEM with 10% fetal calf serum in the presence or absence of 3 mM CPT-cAMP. The nuclei were incubated with ^{32}P-UTP and radiolabeled RNA transcripts isolated as described under "Materials and Methods". The RNA was hybridized to nitrocellulose filters adsorbed with single stranded M13 DNA containing alkaline phosphatase (black bars) or Cα subunit (white bars) cDNA in antisense orientation. Transcription rates were determined from the amount of ^{32}P RNA hybridized to each filter after correction for hybridization efficiencies with an internal control of ^{3}H-labeled Cα subunit RNA.

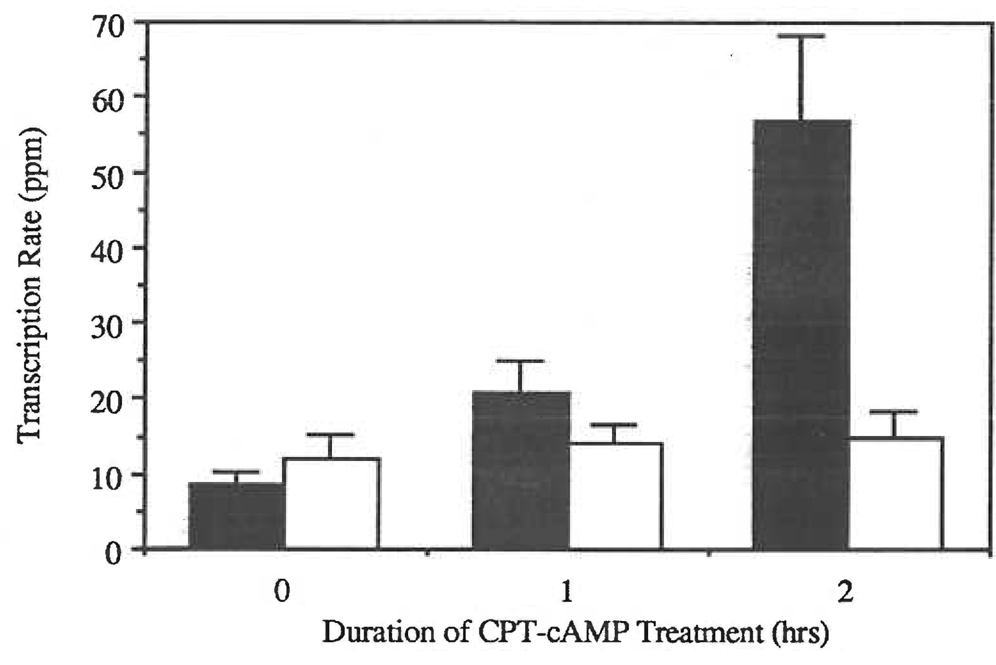
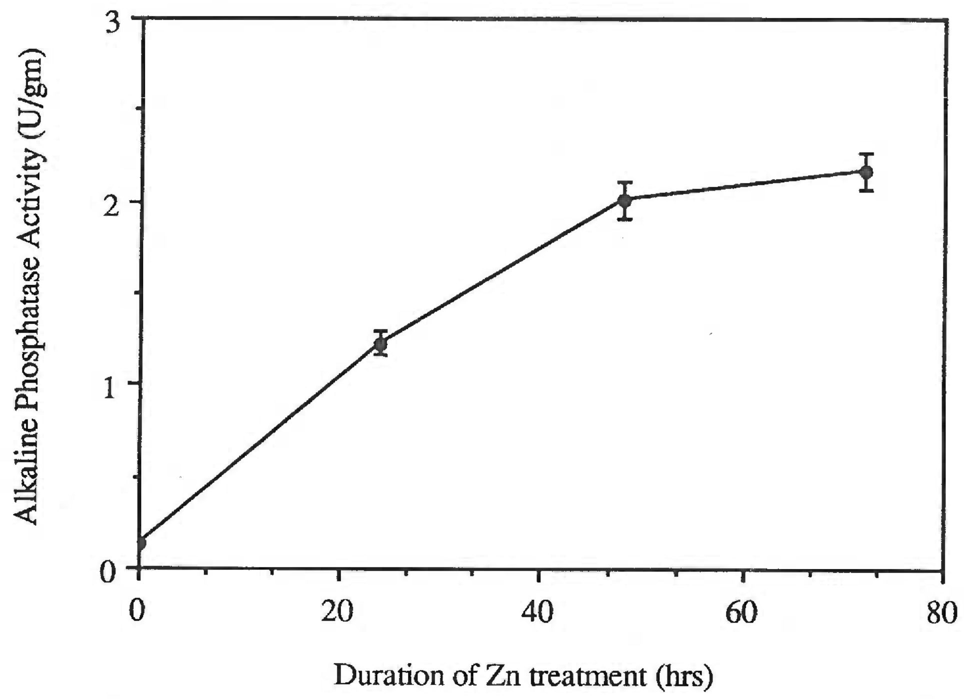

70
60
50
40
30
20
10
0
Transcription Rate (ppm)
0
1
2
Duration of CPT-cAMP Treatment (hrs)

FIG. 5. **The effect of $ZnSO_4$ on alkaline phosphatase activity in Cα2 clone .** L cells were cotransfected with expression vectors for the Cα subunit and neomycin phosphotransferase as described in "Materials and Methods". Mouse L cell clone Cα2 was chosen for further study because of its low level of basal expression and its ability to induce Cα mRNA after Zn treatment. Confluent cultures of clone Cα2 in 10 cm tissue culture dishes were incubated in DMEM containing 10% fetal calf serum and 200 μM $ZnSO_4$. At the times indicated the cultures were harvested, lysates prepared, and assayed for alkaline phosphatase activity and protein content. Each point represents the mean ± S.D. for triplicate determinations.

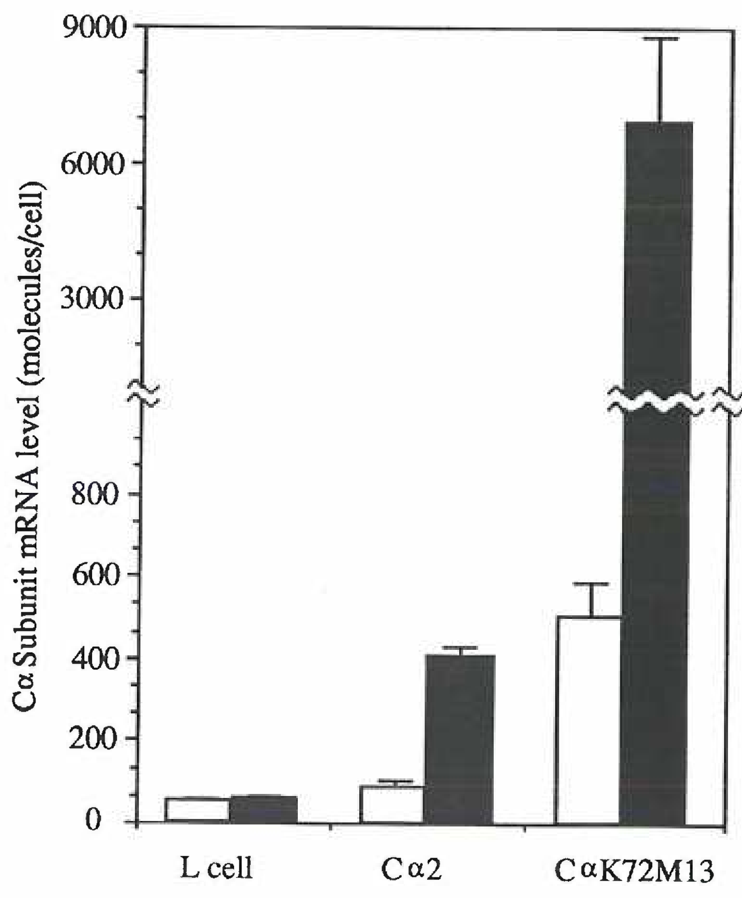


Alkaline Phosphatase Activity (U/gm)
3
2
1
0
0
20
40
60
80
Duration of Zn treatment (hrs)

FIG. 6. **Northern blot analysis of RNA isolated from control, $ZnSO_4$ treated, and CPT-cAMP treated Cα2 cells.** Samples of poly(A) RNA from untreated Cα2 cells, Cα2 cells treated with 200 μM $ZnSO_4$ for 24 h or Cα2 cells treated with 3 mM CPT-cAMP for 24 h were subjected to electrophoresis and hybridization analysis as described under "Materials and Methods". The 2.5 kb mouse L cell alkaline phosphatase cDNA was used to generate ^{32}P-radiolabeled probe. The size of the hybridizing band in the cAMP treated lane was determined by using RNA size standards and staining the gel before transfer to nitrocellulose.

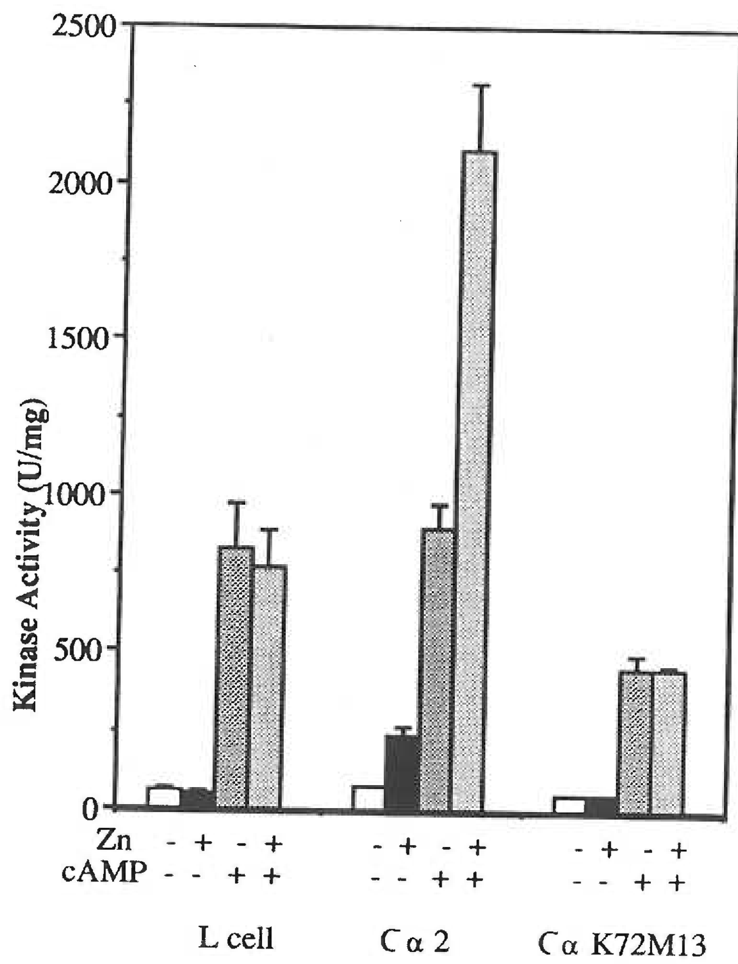
Cα 2
Cα 2 + Zn
Cα 2 + cAMP
— 2.5 kb

FIG. 7. **Effect of $ZnSO_4$ treatment on Cα mRNA levels in mouse L cells, clone Cα2, and clone CαK72M13.** Confluent cultures of mouse L cells, clone Cα2 or clone CαK72M13 were grown in DMEM containing 10% fetal calf serum in the absence (unfilled bars) or presence (filled bars) of 140 µM $ZnSO_4$ for 24 h prior to harvesting for total nucleic acid isolation. Cα mRNA levels were measured as described under "Materials and Methods" using a Cα-specific ^{32}P-labeled SP6 RNA probe in a solution hybridization assay.



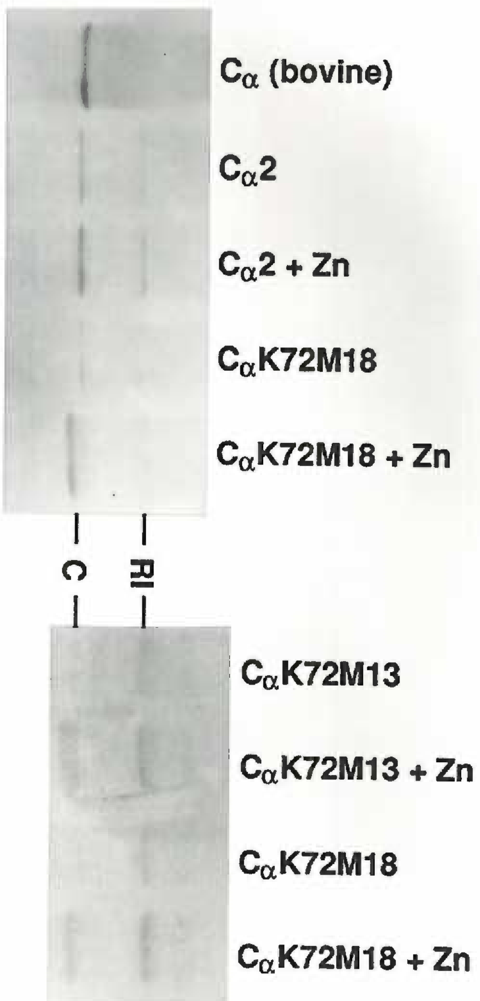
Cα Subunit mRNA level (molecules/cell)
9000
6000
3000
800
600
400
200
0
L cell
Cα2
CαK72M13

FIG. 8. **Effect of $ZnSO_4$ treatment on cAMP-dependent protein kinase levels in mouse L cells, clone Cα2, and clone CαK72M13.** Confluent cultures of mouse L cells, clone Cα2 or clone CαK72M13 were grown in DMEM containing 10% fetal calf serum in the absence (white and hatched bars) or presence (black and stippled bars) of 140 μM $ZnSO_4$ for 24 h prior to harvesting for assay of cAMP-dependent protein kinase. Kinase activity was determined as described in "Materials and Methods" in the absence (black and white bars) or presence (hatched and stippled bars) of 10 μM cAMP. Each value represents the mean ± S. D. for triplicate determinations.



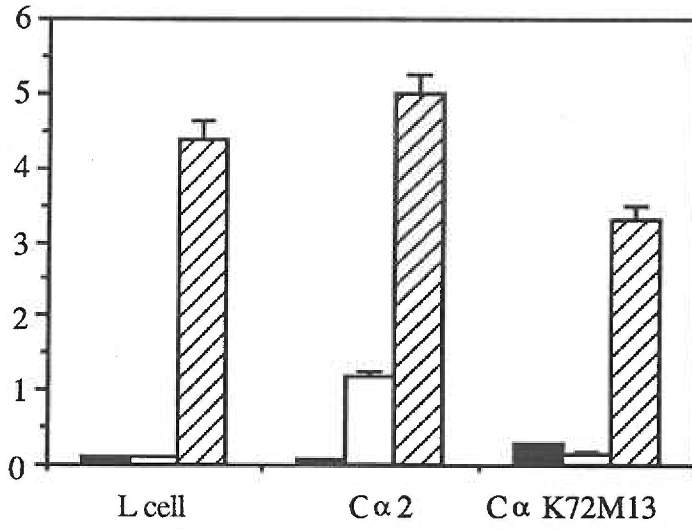
2500
2000
1500
1000
500
0
Kinase Activity (U/mg)
Zn - + - + - + - + - + - +
cAMP - - + + - - + + - - + +
L cell
Cα 2
Cα K72M13

FIG. 9. **Western blot analysis of C and RI subunit proteins in Cα2 and CαK72M18 cells.** Two hundred micrograms of cell extract from Cα2 or CαK72M18 were electrophoresed on a 10% acrylamide and transferred to nitrocellulose. Twenty nanograms of purified bovine heart C subunit was also run for size comparison. The western blot was incubated with a polyclonal antibody against the C subunit followed by an alkaline phosphatase coupled second antibody as described in "Materials and Methods". After color development and identification of the C subunit the blot was further incubated with a polyclonal antibody against the RI subunit.



C_α (bovine)
$C_\alpha 2$
$C_\alpha 2$ + Zn
C_αK72M18
C_αK72M18 + Zn
RI
C
C_αK72M13
C_αK72M13 + Zn
C_αK72M18
C_αK72M18 + Zn

FIG. 10. **The effect of $ZnSO_4$ on alkaline phosphatase activity in mouse L cells, Cα2 cells, and CαK72M13 cells.** Confluent cultures of mouse L cells, Cα2 cells, or CαK72M13 cells were grown in DMEM containing 10% fetal calf serum alone (solid bars), or in the presence of 140 μM $ZnSO_4$ (unfilled bars) or in the presence of 3 mM CPT-cAMP (hatched bars) for 24 h prior to harvesting for assay of alkaline phosphatase activity. Each value represents the mean ± S. D. for triplicate determinations.



Alkaline Phosphatase Activity (U/gm)
6
5
4
3
2
1
0
L cell
C α 2
C α K72M13

Introduction

The growth factor receptor tyrosine kinases are a family of transmembrane proteins characterized by an extracellular ligand-binding domain and an intracellular tyrosine kinase domain (26, 32). This class has been subdivided into three families of proteins which are structurally unique: epidermal growth factor receptor (EGFR), insulin receptor (IR) and platelet derived growth factor receptor (PDGFR). The structure of the EGFR displays an amino-terminal extracellular domain in which N-linked glycosylation is required not only for transport of the receptor to the cell surface but also for EGF binding (16). The rigid ligand-binding domain contains two cysteine-rich sequences and Scatchard analysis of ^{125}I-EGF binding experiments indicates the presence of two distinct states of the EGFR, i.e. a high- and a low-affinity binding state (16, 26). A short transmembrane sequence separates this extracellular domain from a cytoplasmic region which includes the tyrosine kinase domain and a carboxy-terminal autophosphorylation domain (26, 32). The carboxy-terminus consists primarily of small hydrophilic amino acids and is, as a result, extremely flexible. The region connecting this flexible, C-terminal domain to the kinase domain has therefore been called the "hinge region" (16).

EGF binding to the extracellular domain of the EGFR induces a mitogenic cellular response which is accompanied by receptor down-regulation (5, 16, 26, 32). Receptors are known to cluster in coated pits at the cell surface following EGF binding and the receptor-ligand complexes are endocytosed. EGF is then separated from the EGFR and intracellular trafficking moves them along independent paths - the EGF is degraded by lysosomal enzymes, the EGFR is recycled to the cell surface or it, too, is degraded. The cellular response to EGF binding includes Na^+/H^+ exchange, Ca^{+2} influx, S-6 ribosome phosphorylation, c-fos and c-myc expression, macromolecular synthesis (DNA, RNA and protein), inositol triphosphate formation and cell proliferation. Studies of mutant EGFRs and of EGFRs incubated with synthetic inhibitors of EGFR kinase activity have shown that both the mitogenic cellular response to EGF and the normal trafficking of the receptor are

dependent upon the tyrosine kinase activity of the receptor (11, 25, 31). Kinase activity is therefore central to receptor transduction of the extracellular EGF signal.

Activation of the EGFR, as well as that of the other growth factor receptors, follows an ordered mechanism (31, 32):

ligand binding - - - - - > autophosphorylation - - - - - > kinase activation

Although this step-wise process has been recognized, the molecular mechanism by which the tyrosine kinase is activated is unknown (16). One model for activation of the EGFR proposes that the flexible carboxy-terminal autophosphorylation domain of the EGFR may sterically block substrate access to the tyrosine kinase domain and that autophosphorylation of the receptor may result in a conformational change which eliminates this steric interference (32). Kinetic studies of EGFR kinase activity indicate that the autophosphorylation site is itself a competitive inhibitor of exogenous substrate phosphorylation, thereby supporting the proposal that autophosphorylation removes an inhibition of kinase activity (1). A second model of kinase activation is based on recent studies of the EGFR which have shown that EGF binding to the receptor enhances the stability of EGFR dimers (11, 25). In a dynamic equilibrium of EGFR monomers and dimers, it is the dimeric form of the EGFR which exhibits high-affinity binding of EGF (16, 26, 32) and it has therefore been proposed that receptor autophosphorylation is an intermolecular reaction. If the EGFR homodimer is the more active form of the kinase, then kinase activation may be attributed to the receptor oligomerization which follows EGF binding (11, 25, 32).

Because the mechanism of activation of the EGFR kinase is unknown and this kinase activity is essential to transduction of the extracellular signal, we sought to identify amino acid sequences within the EGFR tyrosine kinase domain which are important for enzyme catalysis (Manuscript #2). The 250 amino acids of the kinase domain include a lysine residue (K721 in EGFR) and a glycine triad (G-X-G-X-X-G, residues 695 - 700 in EGFR) which are indicative of the ATP binding site of all protein kinases (16). Alignment

of the EGFR kinase domain with the homologous sequences of other protein kinases has demonstrated a pattern of short stretches of highly conserved sequences interspersed with sequences which are nonconserved, a pattern typical of phylogenetically related globular proteins (13). This pattern of conservation allows the identification of sequences which are likely to be involved in enzyme catalysis or to contribute to maintenance of an active site structure. We have examined four highly conserved sequences in the EGFR kinase domain to determine which, if any, of these amino acids are required for catalysis. Our experimental approach to studying the structure and function of these specific sequences involved the generation of antipeptide antibodies because such antibodies are highly selective in binding to their cognizant sequence within a protein (12, 27). Immunoprecipitation of a native protein by an antipeptide antibody under nondenaturing conditions is evidence that the antibody binding site is exposed on the surface of the protein, thereby indicating the topography of the protein suequence. In addition, if antibody binding to the protein inhibits enzyme activity, this binding has either blocked access to a sequence important for catalysis or has altered the tertiary structure of an important sequence. In either case, such antibody inhibition of catalysis can be used to identify sequences critical to enzyme activity.

The cellular growth factor receptors (EGFR, IR, PDGFR) exhibit regulated tyrosine kinase activity while the tyrosine kinase of the retroviral oncogene products of these receptors is constitutively active (1, 24, 32). The oncogene products represent structurally altered cellular receptors and one of the characteristic alterations is deletion of the growth factor receptor autophosphorylation site (v-erb B/EGFR; v-ros/IR; v-fms/c-fms). Recognition of this consistent pattern of deletion has led to the theory that the autophosphorylation sites of these receptors exert a negative regulation over receptor tyrosine kinase activity through competitive inhibition (16). The flexibility of the carboxy-terminus of the EGFR would allow the autophosphorylation site of the receptor to fold over the kinase domain in a basal, inactive state and autophosphorylation of the receptor could

induce a conformational change which eliminates this carboxy-terminal inhibition of kinase activity (32). The topography of the carboxy-terminus of other growth factor receptors (IR and PDGFR) has already been found to change following autophosphorylation of the receptor as shown by the observation that immunoprecipitation by antibodies to the carboxy-terminal domain produced more receptor protein when the receptor was phosphorylated than when it was not (2, 19, 23). In our studies of EGFR structure and function, we have therefore included antipeptide antibodies to two carboxy-terminal autophosphorylation sites (tyrosines 1068 and 1173).

The cell line chosen for these studies is A431, a human cervical carcinoma cell which expresses about 3×10^6 EGFR per cell (6) as opposed to the fewer than 10^5 per cell seen in human fibroblasts (5). Preparation of A431 membrane vesicles (see Methods of Manuscript #2) yields a concentrated source of EGFR in which about 0.3% of the protein present is EGFR (8). Purification of EGFR from such vesicles, whether by affinity chromatography or by electrophoresis in nondenaturing gels or by immunoprecipitation, has shown that the tyrosine kinase activity, the phosphotyrosine residue and the ability to bind ^{125}I-EGF all reside within the same 170kD protein of this preparation (8). ^{125}I-EGF binds to the EGFR in a 1:1 molar ratio, equilibrium binding is attained in less than 10 minutes at 0^0C and autophosphorylation of the EGFR is complete in about 1 minute (6). The extent of phosphorylation of tyrosine residues of cellular A431 proteins is about 3-fold higher in the presence of EGF than in the absence (17). The EGFR isolated from A431 cell extract, as opposed to A431 membrane vesicles, is primarily in the form of a 150 kD degradation product of the 170kD native protein (8). Although both the 150kD and 170kD proteins possess tyrosine kinase activity, EGF-stimulated autophosphorylation of the 170kD EGFR in A431 membrane vesicles is 5 - 10 times stronger than that observed in the 150kD EGFR of cell extract. This may result from a greater density of receptors in the membrane vesicle preparation or it may be that the 170kD protein is in a more favorable configuration for autophosphorylation than is the 150kD protein.

Structure and Function of Conserved Sites in the Catalytic Domain of the EGF Receptor

Nancy A. Brown, Larry A. Compton, Gail M. Clinton

Abstract

To examine the function and conformation of sites in the catalytic domain of the epidermal growth factor receptor (EGFR), antipeptide antisera were generated against synthetic peptides with sequences derived from the EGFR. Four of these sequences are from highly conserved sites in the catalytic domain and, for comparison, two are nonconserved sequences surrounding the P1 and P3 autophosphorylation sites. Antibodies to all sites were found to specifically immunoprecipitate denatured EGFR that was ligand activated and autophosphorylated. Antisera against two highly conserved sites in the catalytic domain (αALE and αSDV) as well as antisera against the autophosphorylation sites (αP1 and αP3) also immunoprecipitated native, unphosphorylated EGFR and did not inhibit EGFR autophosphorylation in an immunecomplex kinase reaction. Thus epitopes contained in these sequences were exposed on the native protein and did not directly participate in catalysis. In contrast, antisera against two other highly conserved sites (αHRD and αDFG) blocked autophosphorylation of EGFR. Both the native and the denatured autophosphorylated EGFR was immunoprecipitated by αDFG, indicating that epitopes at the DFG site were exposed on the surface of the native protein. The amount of autophosphorylated EGFR immunoprecipitated by αHRD, however, was increased under conditions that opened the protein structure, indicating that the epitopes in the cognate sequence were blocked in the native protein.

Introduction

The epidermal growth factor receptor (EGFR) is one of the most completely characterized examples of receptors with tyrosine kinase activity (5, 16, 26, 32). Activation of the receptor involves ligand binding to an extracellular domain followed by autophosphorylation of cytoplasmic tyrosine residues and activation of the kinase (6, 17, 31). The mechanism by which ligand binding outside the cell triggers activation of the cytoplasmic tyrosine kinase is unknown (16). One theory for the mechanism of activation of EGFR proposes that autophosphorylation causes a conformational change which exposes the catalytic site to exogenous substrates (32). A second explanation is based on the observation that EGF binding to the receptor enhances the stability of receptor homodimers (11). It has been proposed that the dimeric form of the EGFR is more active and that EGF stabilization of dimers results in kinase stimulation (25).

To understand how the kinase is activated and regulated, it may be necessary to define the structure and function of domains and subdomains within the 170kD EGFR. Examination of the molecular architecture of EGFR reveals an N-terminal extracellular domain which is rich in disulfide bonds and contains the ligand binding site. The receptor spans the plasma membrane once and the cytoplasmic region includes a 250 amino acid catalytic domain (16) and a 20 kD carboxy-terminal autophosphorylation domain (26, 32). The autophsophorylation domain is connected to the kinase domain by a flexible "hinge region" and has been proposed to regulate the tyrosine kinase activity of the receptor (16, 32).

The catalytic domain of EGFR as well as other protein kinases can be further divided into subdomains based on the evolutionary conservation of the amino acid sequences. The amino acid residues which are functionally involved in catalysis or which preserve the structural integrity of the active site are conserved (4, 13, 30). Among the functions served by the catalytic domain is binding of ATP. The ATP binding site of protein kinases includes a conserved lysine residue (K721 of EGFR) and a conserved

glycine triad (16, 26, 30). Another conserved sequence within the catalytic domain is the tripeptide DFG (4) which is invariant among 64 of the 65 kinases whose amino acid sequences have been aligned (13). The aspartate residue of the DFG triad as well as that of the conserved HRD triad have been proposed to be important in stabilizing ATP binding thru magnesium salt bridges (3). Recognition of the proper hydroxyamino acid as the distinguishing feature of the substrate protein is another function of the catalytic domain of protein kinases. The substrate binding site of protein tyrosine kinases may be different from that of serine/threonine kinases to accomodate the tyrosine and would therefore be expected to be conserved primarily among members of the tyrosine kinase family of proteins. Both LAAR (of the HRD peptide) and PIKWM (of the ALE peptide) are sequences from the human EGFR (residues 814 - 817 and 858 - 860) which are preferentially conserved in the tyrosine kinases (4, 13).

Both site-directed mutagenesis and antipeptide antibodies have been of value in examining protein kinase structure and function. Antipeptide antibodies can be used to examine the topography of a specific tyrosine kinase sequence because immunoprecipitation of the denatured protein but not the native protein is evidence that the specific amino acid sequence to which the antibody binds is buried in the native protein. For example, antibody generated against the major autophosphorylation sites of the insulin receptor (IR) immunoprecipitates only the phosphorylated receptor, suggesting that autophosphorylation exposes the site that contains this tyrosine residue (23). Topological studies of the platelet derived growth factor receptor (PDGFR) have shown that antibody against the carboxy-terminal region immunoprecipitates about nine times more protein when the receptor is phosphorylated (19). As with the insulin receptor, autophosphorylation induces a change in receptor topography.

In addition to topographical studies, antipeptide antibodies have been used to examine whether an amino acid sequence is critical for kinase activity. Inhibition of phosphotransferase activity following antibody binding is evidence of the importance of the

sequence for catalysis. Antibody to a potential autophosphorylation site (Y960) in the juxta-membrane region of the IR β subunit inhibits both autophosphorylation and protein tyrosine kinase activity (14). Antipeptide antibody binding to a sequence homologous to the autophosphorylation site of pp60 src in the v-abl product (20) and in the IR and EGFR (18) inhibits kinase activity. Phosphorylation of this tyrosine (Y1150) in the IR enhances tyrosine kinase activity (15, 32), but phosphorylation in the EGFR has not been demonstrated.

Because antipeptide antibodies can be used to probe specific protein sequences, we chose this approach to examine the topography and functional importance of six amino acid sequences in the EGFR: four sites are highly conserved within the kinase domain and may be important for catalysis; two (P1 & P3) are autophosphorylation sites in the carboxy-terminal autophosphorylation domain and have been suggested to be involved in regulation of kinase activity (1, 15, 24, 32).

Materials & Methods

Peptide synthesis and immunization of rabbits - Peptides were syntesized by the solid phase method (9, 10) using PepSyn KA resin and Fmoc amino acid esters (Milligen). After cleavage from the resin, the peptide was analyzed by HPLC and amino acid analysis of trypsin cleavage products. Peptides were conjugated to hemocyanin, rabbits were immunized and test bled as described (7).

Cell culture and A431 membrane vesicle preparation - A431 cells (ATCC CRL 1555) were cultured in Modified Eagle's Medium containing 5% fetal bovine serum (Gibco) and 0.5% gentamicin (Gibco). A431 membrane vesicles were prepared by incubation of the cells in roller bottles with a hypotonic vesiculation buffer as described by Cohen (8) and as modified by Lin & Clinton (21). This preparation was stored at -70^{0}C in 10mM HEPES, pH 7.4 at 2 - 4 mg/ml protein. Protein concentrations were determined spectrophotometrically using a BioRad dye binding assay.

Ligand activation and in vitro autophosphorylation - A431 membrane vesicles were incubated for 10 minutes at room temperature in 0.67uM EGF (Collaborative Research) to allow ligand binding. Each immunoprecipitation was performed using 2.5 ug of ligand-activated A431 protein.

For autophosphorylation of EGFR prior to immunoprecipitation, 25 ug of the EGF-bound A431 vesicle protein was incubated for 10 minutes on ice in a 100 uL kinase reaction mixture (20mM HEPES, pH 8, 2mM dithiothreitol, 0.5% NP-40, 25uM $NaVO_4$, 10uM $MnCl_2$) containing 10uM ATP and 0.2uM (γ-^{32}P) ATP (6000 Ci/mmole, NEN). 2.5 ug of this autophosphorylated EGFR was used in each immunoprecipitation.

For autophosphorylation of EGFR following immunoprecipitation, the immunecomplex was washed twice in the same buffer (A, B or C) used for immunoprecipitation and then incubated for 10 minutes on ice in 100 uL of a kinase reaction mixture containing 10uM ATP and 0.5uM (γ-^{32}P) ATP.

Immunoprecipitation - Binding of antibody to EGFR was performed for 5 minutes in a water bath at 34^0C, then for 55 minutes on a rotating platform at 4^0C. The binding and immunoprecipitation took place in one of three solutions (A, B, C):

A) 20mM HEPES, pH 7.6, 1% Triton X-100, 10% glycerol, 2mM Na_3VO_4, 1% aprotinin, 1mM PMSF

B) Solution A plus 150mM NaCl and 0.05% SDS

C) 10mM Tris HCl, pH 7.6, 150mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 2mM $NaVO_4$, 1% aprotinin, 1mM PMSF

The antibody-EGFR complex was adsorbed to 100 ul of a 50% suspension of protein A sepharose (Sigma) for 45 minutes at 4^0C on a rotating platform. The immunecomplex was washed 2X in whichever solution had been used for immunoprecipitation, autophosphorylation in the immunecomplex could be performed as described above, and the immunecomplex was washed 4X in solution C. The proteins in the immunecomplex were eluted using 100 ul of Laemmli reducing sample buffer (4% SDS, 40% glycerol, 0.125M Tris, pH 6.8, 2% β–mercaptoethanol, 0.1% bromophenol blue) heated to 100^0C for 2 minutes. These proteins, along with SDS-PAGE molecular weight standards (BioRad), were electrophoresed (7.5% acrylamide) and radiolabeled EGFR was identified by autoradiography using Kodak X-Omat K film.

Results

Generation and characterization of antipeptide antibodies

To study the function and topography of specific sites in the EGFR, we synthesized four peptides with sequences from the catalytic domain of the EGFR and two with sequences from the autophosphorylation domain. The relative positions of these sequences within the human EGFR are shown in Fig. 1 and the complete amino acid sequence of each peptide is given in Table I. The peptides were conjugated to hemocyanin and rabbits were immunized to generate antipeptide antibodies.

Antipeptide antibody production in the immunized rabbits was monitored by testing the sera for immunoprecipitation of EGFR. The EGFR was from membrane vesicles prepared from A431 cells, a human cervical carcinoma cell line expressing elevated levels of EGFR (6, 8). Prior to immunoprecipitation, the A431 vesicles were incubated with EGF and added to a kinase reaction mixture (described in Materials and Methods) containing (γ-^{32}P) ATP. The radiolabeled, autophosphorylated EGFR from these vesicles was then immunoprecipitated in a denaturing buffer containing 0.1% SDS and 1% deoxycholate (buffer C in Methods). For comparison, immunoprecipitation assays were performed in parallel with a well-characterized standard antibody which binds to the extracellular domain of the EGFR (21). Each of the six antipeptide antisera immunoprecipitated a radiolabeled 170kD protein which comigrated on a 7.5% polyacrylamide gel with the radiolabeled EGFR immunoprecipitated by the standard antibody (data not shown). The 170kD protein was not immunoprecipitated by preimmune sera from each of the rabbits.

The antibodies were also characterized by ELISA using immobilized peptide. Although the ELISAs were positive, the titer did not correlate with the immunoprecipitation titer. This discrepancy between titer by immunoprecipitation of the whole protein and titer by ELISA using immobilized peptide has been reported by others and has been attributed to

differences in conformation of the isolated peptide and the conformation assumed by the amino acid sequence in the protein (28).

The sequence specific binding of the antipeptide antibodies to sites in the EGFR was examined by two methods. Immunoprecipitation of the EGFR by antibodies directed against the P1, P3, HRD and DFG sequences was blocked by preincubating the antibody with the synthetic peptide. The IgGs precipitated by ammonium sulfate from the antisera against the HRD, ALE and SDV sequences were shown to specifically bind to an Affigel 10 peptide affinity column (data not shown).

Effects of denaturing agents on immunoprecipitation of EGFR by antipeptide antibodies

To investigate the topography of specific sites in the EGFR, immunoprecipitation of the receptor by the antipeptide antibodies was performed in buffers A, B, C whose denaturing properties varied (buffers described in Methods). Buffer A contains only nonionic detergents and is the least denaturing. Therefore, only epitopes normally found on the surface of the native protein are likely to be exposed when the EGFR is suspended in buffer A. Tyrosine kinase activity was maintained in buffer A, both before and after immunoprecipitation (data not shown). Buffer B contains 0.05% SDS and was used to partially open the structure of the protein while maintaining kinase activity. In buffer B, autophosphorylation of the EGFR in the immunecomplex was at least 90% of that observed in buffer A (see Fig. 2). Buffer C, however, contained a higher SDS concentration (0.1%) as well as 1% of the ionic detergent deoxycholate. Suspension of the EGFR in buffer C resulted in complete inhibition of kinase activity. Buffer C was used to expose epitopes that may be buried in the native EGFR and was therefore of value when assessing the titer of the antipeptide antibodies in immunoprecipitation of the EGFR. The standard antibody, used as a positive control, binds to the extracellular domain of the EGFR and immunoprecipitates more than 90% of the native or denatured EGFR under conditions of antibody excess (21 and unpublished observations).

Because the topography of major autophosphorylation sites in the IR as well as in the carboxy-terminal domain of the PDGFR changes following receptor autophosphorylation (2, 19, 23), we first examined the topography of the major autophosphorylation site, P1 (Y1173) of the EGFR. Immunoprecipitation of EGFR by αP1 antibody was conducted in each of the three buffers (A, B, C) and a similar amount of ligand-activated, autophosphorylated EGFR was immunoprecipitated each time (Fig. 2, lane 1). The exposure of the P1 site in the native EGFR was therefore equal to that in the denatured receptor. In addition, the ligand-bound, unphosphorylated receptor was immunoprecipitated and then autophosphorylated (postphosphorylation) in the immunecomplex (Fig. 2, lane 2). Equal amounts of receptor postphosphorylation were observed in buffers A and B, but autophosphorylation in the immunecomplex was not detected in buffer C because the kinase had been inactivated by the detergents in this buffer. The amount of autophosphorylated EGFR that was immunoprecipitated relative to the amount that was autophosphorylated in the immunecomplex in buffers A and B was comparable to what was observed with the standard antibody, indicating that the exposure of the P1 sequence was not dependent on the phosphorylation state of the receptor and that binding of αP1 antibody did not inhibit autophosphorylation, despite the fact that antibody was bound at or near the autophosphorylation site. When αP3, antibody generated to the third autophosphorylation site (Y1068) was used, equivalent amounts of pre- and postphosphorylated EGFR were again observed (data not shown). It appears, therefore, that epitopes contained in sequences surrounding P1 and P3 were exposed in both the unphosphorylated and autophosphorylated EGFR and that denaturation did not enhance exposure at these sites.

Immunoprecipitation of the autophosphorylated EGFR in buffers A, B and C was also compared using the four antipeptide antibodies to the kinase domain.. With the exception of the αHRD antibody, there was nearly equivalent exposure of the cognizant sequences (DFG, ALE, SDV) in all three buffers. For the αHRD antibody, however,

there was very little EGFR immunoprecipitated in buffer A under standard conditions of immunoprecipitation. The efficiency of immunoprecipitation was significantly increased, however, by the addition of 0.05% SDS to the buffer (Fig. 3). In addition, if the autophosphorylated EGFR was incubated with αHRD briefly at an elevated temperature (5 minutes at 34^{0}C), there was a two-fold increase in the amount of receptor that was immunoprecipitated (Fig. 3). It appeared that the HRD site was relatively inaccessible to antibody binding in the native protein (no SDS) at 4^{0}C, but a partial opening of the protein resulted in increased exposure of the HRD sequence to antibody binding. In contrast to the other conserved sites in the catalytic domain, HRD appeared to be buried or blocked in the native protein.

Immunoprecipitation of phosphorylated and unphosphorylated EGFR

To assess whether antibodies immunoprecipitate unphosphorylated EGFR and to examine autophosphorylation in the immunecomplex, A431 membrane vesicles were incubated with EGF, the unphosphorylated EGFR was immunoprecipitated by each of the antipeptide antibodies and the immunecomplex was incubated in a kinase reaction mixture containing (γ-^{32}P) ATP. Immunoprecipitation of unphosphorylated EGFR was conducted in parallel with autophosphorylated, ^{32}P labeled A431 vesicles. Proteins were eluted from the immunecomplexes, resolved by SDS-PAGE and the gels were autoradiographed to identify and quantitate radiolabeled EGFR. While all six (see Fig. 2 for αP1) of the antipeptide antibodies immunoprecipitated the prephosphorylated EGFR (Fig. 4, lane 1), two antibodies to conserved sites in the catalytic domain (αALE and αSDV) as well as the antibodies to the autophosphorylation sites (αP1 and αP3) also immunoprecipitated the unphosphorylated EGFR (Fig. 4, lane 2) and binding of antibody to these sequences did not inhibit autophosphorylation in the immunecomplex. In contrast, reduced levels of autophosphorylation in the immunecomplex was observed following immunoprecipitation of unphosphorylated EGFR with αHRD. Moreover, the autophosphorylation observed in

the αDFG immunecomplex was significantly less than that seen with the αALE, αSDV, αP3 and αP1 antibodies. A similar result was obtained using antisera from several different rabbits immunized with the HRD and DFG peptides.

Effects of αHRD and αDFG on autophosphorylation of EGFR

We designed experiments to further explore whether αHRD and αDFG, while bound to their cognate sequences, blocked autophosphorylation of EGFR. For these studies, a standard antibody was employed which does not inhibit autophosphorylation of EGFR in the immunecomplex (Fig. 5, lane 2). Because the unphosphorylated EGFR may not bind to αHRD and αDFG (Fig. 5, lane 2), we also used each of the antibodies to immunoprecipitate EGFR autophosphorylated with (γ-^{32}P) ATP to demonstrate that EGFR was present in the immunecomplex (data not shown). The ^{32}P-labeled immunecomplex was treated with prostatic acid phosphatase, a phosphotyrosyl phosphatase (21), to dephosphorylate tyrosines and provide substrate for the autophosphorylation reaction. Dephosphorylation was incomplete, since about 70% of the ^{32}P was retained in the EGFR treated with phosphatase (Fig. 5, lane 3) compared to the control radiolabeled EGFR which was not incubated with phosphatase (data not shown). To test whether the antipeptide antibodies block autophosphorylation of this dephosphorylated EGFR, EGFR which had been prephosphorylated with unlabeled ATP was immunoprecipitated as above, dephosphorylated in the immunecomplex with the phosphatase and then incubated in a kinase reaction mixture containing (γ-^{32}P) ATP. While the dephosphorylated EGFR bound to the standard antibody was autophosphorylated in the immunecomplex with (γ-^{32}P) ATP (Fig. 5, lane 1), there was no ^{32}P incorporated into the dephosphorylated EGFR that was bound to either αHRD or αDFG (Fig. 5, lane 1), indicating that autophosphorylation was blocked by both antipeptide antibodies.

Discussion

Antipeptide antisera generated against six amino acid sequences from the human EGFR were shown to bind their cognate sequences and to immunoprecipitate denatured, autophosphorylated EGFR. Three antisera to sites from the catalytic domain (αDFG, αALE and αSDV) and two against the carboxy-terminal autophosphorylation domain (αP1 and αP3) immunoprecipitated equal amounts of EGFR regardless of the denaturing strength of the buffer used, while one antiserum against the catalytic domain (αHRD) required at least 0.05% SDS or incubation at an elevated temperature (34^{0}C) for immunoprecipitation to occur. Antibodies to the ALE, SDV, P3 and P1 sequences of the EGFR bound to the unphosphorylated EGFR as well as to the phosphorylated receptor and this binding did not inhibit autophosphorylation in the immunecomplex. Binding of antibody to the HRD and DFG sequence, on the other hand, blocked autophosphorylation in the immunecomplex.

The observation that αHRD and αDFG inhibited EGFR autophosphorylation in the immunecomplex provides experimental evidence for the proposed functional importance of two highly conserved protein kinase sequences, HRD and DFG. A computer template designed by S. Brenner (3) to search for proteins exhibiting phosphotransferase activity contained only three elements - a hystidine, an aspartate and an asparagine at specific positions relative to each other:

H-X-**D**-X_4-**N**-3 hydrophobic amino acids

This template identified bacterial phosphotransferases as well as eukaryotic protein kinases. While searching the database, it was found that all the proteins identified by the template also contained another aspartate (3). In the eukaryotic protein kinases, this second aspartate is usually 13 residues downstream from the first (13). These invariant aspartates and asparagine constitute the pattern:

D-X_4-**N**-X_{13}-**D**

Examination of the amino acid sequence of the human EGFR revealed to us that the conserved HRD and DFG sequences fit this pattern exactly (see Table I). Such stringent sequence conservation implies that these residues perform a critical function common to all phosphotransferases. It has been suggested that the two conserved aspartate residues may be important in ATP binding, i.e. that Mg-ATP salt bridges could bind the nucleotide to the anionic aspartate residues of the kinase domain (3). It has also been proposed that the mechanism by which phosphate transfer actually takes place may involve a nucleophilic residue, possibly an aspartate (30). This nucleophile may enhance the nucleophilic properties of the phosphate-accepting hydroxyamino acid, for example, the tyrosine residue of the EGFR substrate. In fact, the aspartate residue of the highly conserved DFG sequence has been suggested to be the nucleophile which initiates this phosphotransferase reaction (29).

The four highly conserved sequences which we examined in the catalytic domain are positioned within 70 amino acids of each other in the primary amino acid sequence of the EGFR (Fig. 1). Thus the neutralization of kinase activity by antipeptide antibodies showed exquisite site-specific effects despite the large size of the IgG compared to the peptide sequence to which it binds. Others working with site-specific antibodies have observed similar precision in sequence-specific effects of the antibody. For example, two antipeptide antibodies against sequences in pp60 src which are separated by only eight amino acids differ in their ability to block kinase activity (12). Because the binding sites are so close to each other, the inhibition of kinase activity by one of the antibodies and not the other was attributed to interaction of the antibody with a specific, functionally-important sequence in the catalytic domain of pp60 src rather than to a gross steric effect.

Results presented here along with previous studies using antipeptide antibodies (18) indicate that the following catalytic domain sequences are important for EGFR kinase activity:

^{811}HRDLAARN--------VKITDFGLA----AEEKEYHAEGGK^{851}

The first is the HRD sequence, the second is the DFG sequence and the third is the human EGFR homologue (Y845) of the pp60 src autophosphorylation site. However, αALE and αSDV antiserum did not inhibit EGFR autophosphorylation. The synthetic peptide which we had used to generate the αALE antibody (see Table I) is the sequence immediately downstream from the pp60 src autophosphorylation homologue in the human EGFR, yet this antibody did not block autophosphorylation. While mutagenesis of single amino acids within the APE sequence of pp60 src (homologous to ALE residues 858 - 860 of EGFR) results in diminished kinase activity (4), the primary effect of the mutagenesis may have been alteration of the tertiary structure rather than elimination of an amino acid which is required for catalysis. Because antibody binding to the ALE and SDV sites did not block catalytic activity , we conclude that either these sequences are conserved because they are required to maintain a particular protein structure or that our antibodies did not neutralize the amino acids at these sites which participate in catalysis.

Having identified sequences within the catalytic domain that are important for catalysis, it remains to be determined how EGFR kinase activity is regulated. The accessibility of catalytically important amino acids may play a role in this regulation. The DFG sequence of the autophosphorylated EGFR was as exposed in non-denaturing buffer A as it was in buffer C which contained SDS and deoxycholate, indicating that this site is accessible in the native protein. The HRD epitope of the phosphorylated EGFR, however, was relatively inaccessible to antibody binding. Whether the exposure of these sequences changes following EGF binding to the receptor or autophosphorylation is an important question which remains to be answered.

The two autosphorylation sites P1 and P3 were exposed in the native EGFR (buffer A) and denaturation in buffer C did not increase the efficiency of immunoprecipitation. Moreover, the extent of immunoprecipitation was not dependent on the phosphorylation state of the receptor because similar quantities of phosphorylated and unphosphorylated EGFR were immunoprecipitated. This topography of the autophosphorylation sites in the

EGFR is different from some other members of the growth factor receptor tyrosine kinase family. Antibody to the major autophosphorylation site of the insulin receptor immunoprecipitates the phosphorylated, but not the unphosphorylated, receptor (23) and antibody to the carboxy terminus of the platelet derived growth factor receptor immunoprecipitates 9-fold more protein if the receptor is phosphorylated (19). The carboxy terminus of the EGFR appears to be exposed and this may explain the intermolecular autophosphorylation of EGFR dimers which has been observed (25).

Antibody binding to the autophosphorylation site, whether it was P1 or P3, did not inhibit autophosphorylation. It has also been found that antibody binding to the carboxy terminus of the platelet derived growth factor receptor does not neutralize autophosphorylation (2) and antibody to the sequence just downstream from P1 (1175 - 1186) does not inhibit EGFR autophosphorylation (18). Either the tyrosines to be phosphorylated in these cases did not directly bind to antibody or the hydroxyl group was accessible to the catalytic site of the kinase despite the presence of antibody. In contrast, antibody binding to the homologue of the src autophosphorylation site in the kinase domain of the EGFR and IR inhibits autophosphorylation (18), even though this site has not been found to be autophosphorylated in the EGFR. Thus the autophosphorylation sites within the kinase domain appear to play a direct or regulatory role in catalysis.

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Conclusion

These studies using antibodies that bind to specific amino acid sequences of the EGFR identified the following structural and functional features of this growth factor receptor tyrosine kinase:

1. The ALE and SDV sequences of the EGFR kinase domain are exposed in the native receptor and this exposure is not influenced by the phosphorylation state of the EGFR. These sites in the kinase domain do not appear to be involved in enzyme catalysis.

2. The carboxy-terminal autophosphorylation sites (Y1068 and Y1173) of the EGFR are exposed in both the unphosphorylated and the autophosphorylated EGFR. This exposure does not change with receptor autophosphorylation, a condition which is unlike that observed with carboxy-terminal sites of other growth factor receptor tyrosine kinases (i.e. insulin receptor and platelet derived growth factor receptor). These autophosphorylation sites do not appear to be involved in catalysis.

3. The HRD and DFG sequences of the tyrosine kinase domain appear to be important for catalytic activity because antibody binding to these sites inhibits receptor autophosphorylation.

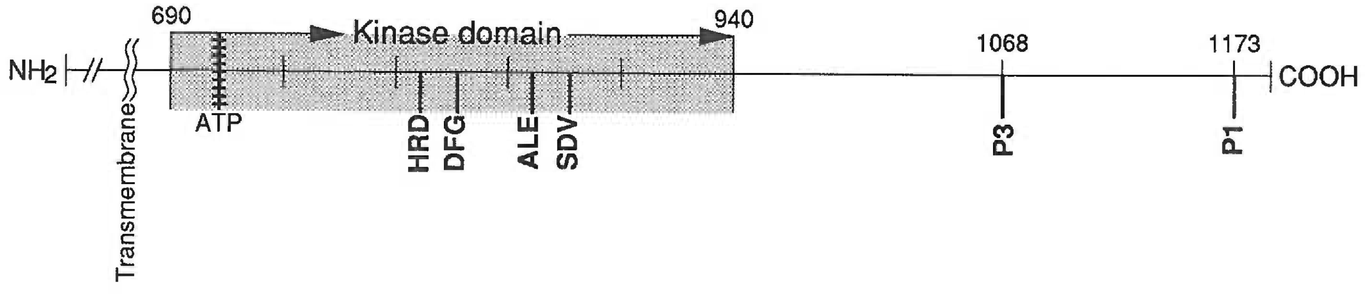
4. The HRD site in the EGFR kinase domain appears to be either buried in the native protein or blocked because the nondenatured protein had to be opened slightly by 0.05% SDS or by incubation at 34^0C to allow antibody binding. This was not true of any other sequence examined, either in the kinase domain or in the autophosphorylation domain.

The topography of the EGFR has not been elucidated and the mechanism of phosphotransferase activity remains unclear. Because an antipeptide antibody binds to a specific protein sequence, the approach we have used allows differentiation between the relative exposure and functional importance of selected sequences within the protein. After identification of functionally important protein sequences, such as the HRD and the DFG sequences in the EGFR tyrosine kinase domain, the relative exposure of these sequences

becomes important because of the potential role this exposure plays in regulation of catalysis, i.e. phosphotransferase activity. The aspartate residues of both the HRD and DFG sequences have been proposed to be involved in ATP binding to protein kinases (3, 23, 29) and the LAAR sequence of the HRD peptide may be involved in tyrosine kinase substrate recognition and binding (13). The accessibility of these two sequences for nucleotide binding or substrate binding is therefore important to kinase function. Whether EGF binding to the EGFR or autophosphorylation of the receptor affects the exposure of either of these two sequences remains to be investigated.

Fig. 1 **Position of Peptide Sequences in Human EGFR**

Amino acids are numbered from the amino terminus of the EGFR. The conserved lysine (K721 in EGFR) is indicated as the ATP binding site.



690
Kinase domain
940
1068
1173
NH2
COOH
Transmembrane
ATP
HRD
DFG
ALE
SDV
P3
P1

Fig. 2 **Detergent effects on αP1 antibody binding**

αP1 antiserum was used to immunoprecipitate ligand-activated EGFR from A431 membrane vesicles in one of three buffers A, B, or C (contents of buffers described in Methods). Lane 1, immunoprecipitation of autophosphorylated, P-32 labeled EGFR; Lane 2, immunoprecipitation of unphosphorylated EGFR followed by incubation of the immunecomplex in a kinase reaction mixture containing (γ-^{32}P) ATP (see Methods).

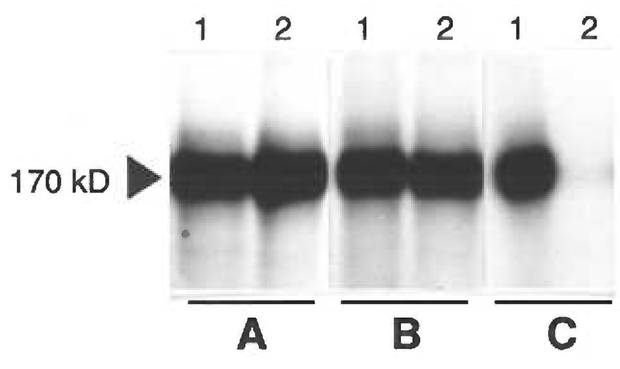

1
2
1
2
1
2
170 kD
A
B
C

Fig. 3 **Effects of detergent and temperature on αHRD antibody binding** αHRD antiserum was incubated with ligand-activated, autophosphorylated A431 membrane vesicles on a rotating platform: Lane 1, 5 minutes at $34^{0}C$ followed by 55 minutes at $4^{0}C$; Lane 2, one hour at $4^{0}C$. The solution used for immunoprecipitation contained either 0.05% SDS or 0% SDS (buffers B and A in Methods).

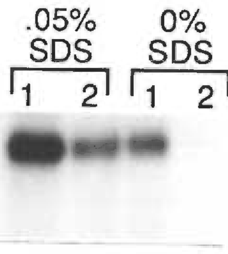

.05%
SDS
1
2
0%
SDS
1
2

Fig. 4 **Immunoprecipitation of EGFR by antipeptide antibodies**

EGFR was immunoprecipitated in buffer B (see Methods) from ligand-activated A431 membrane vesicles by each of the antipeptide antibodies. Lane 1, immunoprecipitation of autophosphorylated, P-32 labeled EGFR; Lane 2, immunoprecipitation of unphosphorylated EGFR followed by incubation of the immunecomplex in a kinase reaction mixture containing (γ-^{32}P) ATP (see Methods).

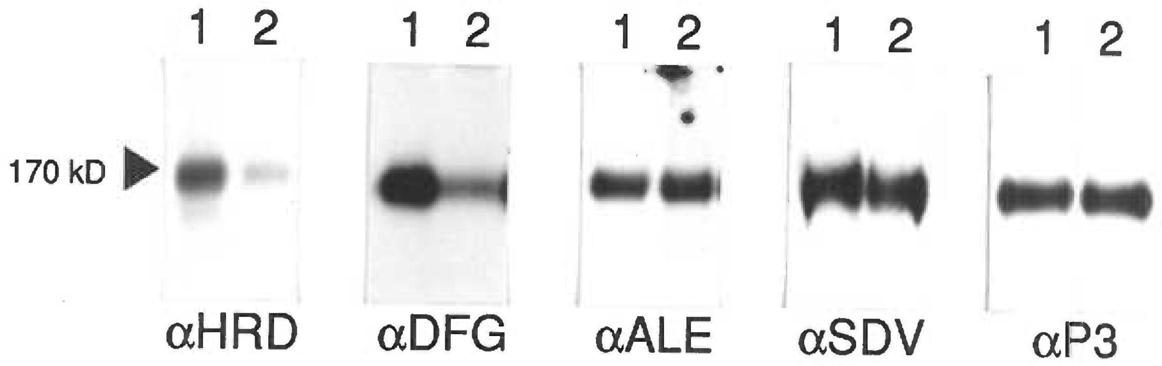
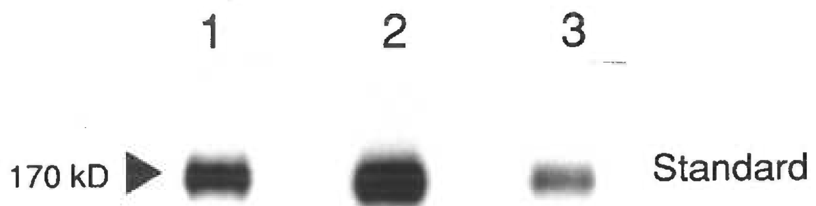
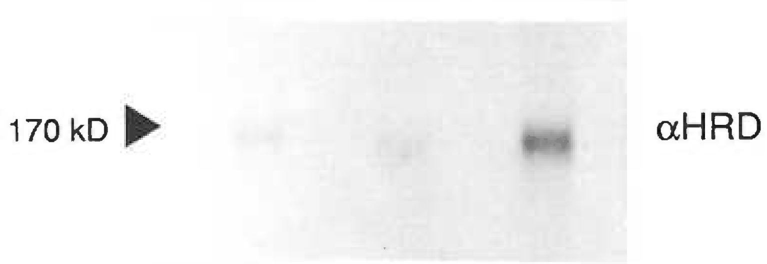

1 2
1 2
1 2
1 2
1 2
170 kD
αHRD
αDFG
αALE
αSDV
αP3

Fig. 5 **Effects of αHRD or αDFG binding on autophosphorylation of EGFR** Ligand-activated A431 membrane vesicles were incubated for 10 minutes on ice in a kinase reaction mixture containing: Lane 1, 10uM ATP; Lane 2, no ATP; Lane 3, 10uM ATP + 0.2uM (γ-^{32}P) ATP (6000 Ci/mmole, NEN). EGFR was then immunoprecipitated in buffer B (see Methods) and the immunecomplex was washed twice in buffer A. 2 ug of purified prostatic acid phosphatase in 100 ul of buffer A was added to each sample and incubated with the immunecomplex for 10 minutes at 24^{0}C. After washing each immunecomplex twice with buffer A, 75 ul of a kinase reaction mixture was added and the sample incubated on ice for 10 minutes. The kinase reaction mixtures contained: Lanes 1 and 2, 10uM ATP + 0.5uM (γ-^{32}P) ATP; Lane 3, no ATP. Proteins were eluted from the immunecomplex in Laemmli sample buffer, electrophoresed in SDS-PAGE and the dried gels were autoradiographed.


1
2
3
170 kD
Standard


170 kD
αHRD

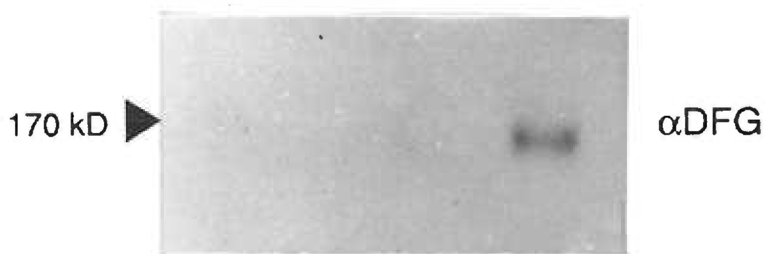

170 kD
αDFG

Table I **Complete amino acid sequence of synthetic peptides**

The peptide sequences were taken from the human EGFR. Amino acid residues are numbered starting from the amino terminus of the protein.

	Peptide Sequence	Position in Human EGFR
HRD	HRDLAARN	811- 818
DFG	VKITDFGLA	827- 835
ALE	VPIKWMALES	852- 861
SDV	SDVWSYGV	871- 878
P3	DDTFLPVPEYINQS	1059-1072
P1	TAENAEYLRVAPQS	1167-1180