

Protein kinase C modulates the up-regulation of the pyrimidine biosynthetic complex, CAD, by MAP kinase

Frederic D Sigoillot², Damian H. Kotsis², Elizabeth M. Masko², Monica Bame¹, David R. Evans², Hedeel I. Guy Evans¹

¹Department of Chemistry, Eastern Michigan University, Ypsilanti, Michigan, 48197, ²Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan, 48201

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Material and methods
 - 3.1. Cell culture methods
 - 3.2. Preparation of cell extracts
 - 3.3. Enzyme assays
 - 3.4. Immunoblotting
 - 3.5. Cloning and expression of CAD and GLN-CPSase
 - 3.6. Transfection
4. Results
 - 4.1. Phosphorylation of CAD by protein kinase C
 - 4.2. Effect of PKC activation in vivo
 - 4.3. Effect of kinase inhibitors on PMA induced CAD phosphorylation
 - 4.4. CAD phosphorylation in EGF stimulated cells
 - 4.5. CAD deletion mutant
5. Discussion
6. Acknowledgements
7. References

1. ABSTRACT

The multifunctional protein CAD initiates *de novo* pyrimidine biosynthesis in mammalian cells. CAD is activated by MAP kinase (Erk1/2) just prior to the S phase of the cell cycle, when the demand for pyrimidine nucleotides is greatest, and down-regulated as the cells emerge from S phase by protein kinase A (PKA) phosphorylation. MAP kinase phosphorylates Thr456, while PKA phosphorylates Ser1406 and Ser1859, although only Ser1406 is involved in regulation. LC/mass spectrometry showed that Ser1873, a residue that lies within a putative protein kinase C (PKC) consensus sequence is also phosphorylated. Purified CAD was reacted with ATP and a panel of eight PKC isozymes. Most isozymes resulted in limited CAD phosphorylation, but the delta and epsilon isozymes were most effective. While the level of Thr456 phosphorylation is very low in

confluent cells, exposure of stationary BHK 165-23 cells to the PKC activator, phorbol 12-myristate-13-acetate (PMA) resulted in a 3-fold increase in the modification of this residue. The stimulation of Thr456 phosphorylation was blocked by PKC inhibitors. The PKA inhibitor, H-89, also stimulated PMA-induced Thr456 modification probably because PKA mediated phosphorylation of CAD Ser1406 antagonizes the MAP kinase phosphorylation. Thus, the extent of Thr456 phosphorylation and the activation of pyrimidine biosynthesis depend on the synergistic and antagonistic interactions of three signaling pathways, MAP kinase, PKC and PKA. Deletions mutants lacking the putative PKC site, Ser1873 do not exhibit PMA induced Thr456 phosphorylation. We conclude that the activating MAP kinase phosphorylation of CAD proceeds through a PKC dependent pathway.

PKC promotes MAP kinase phosphorylation of CAD

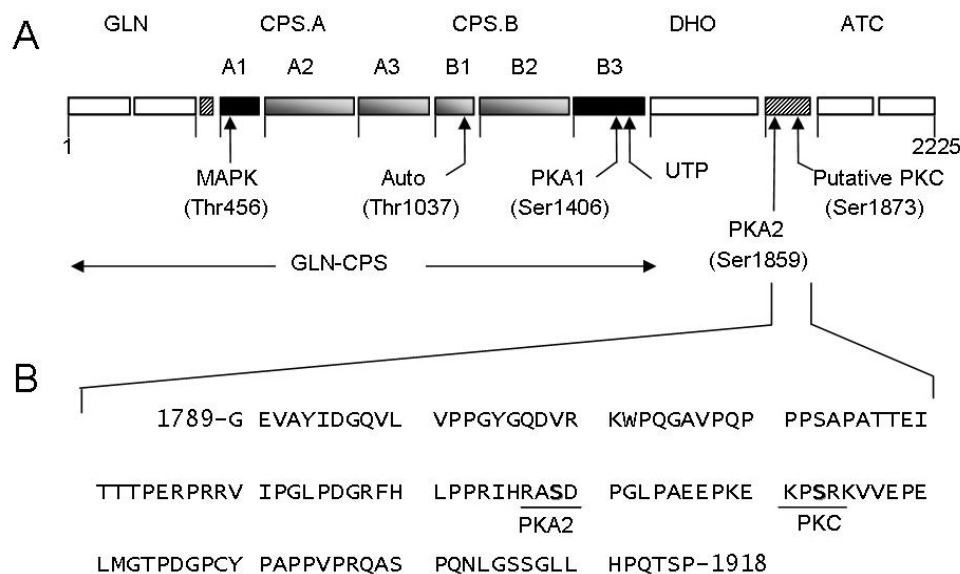


Figure 1. CAD domain structure and phosphorylation sites. *Panel A:* The schematic shows the functional domains of CAD each of which consists of several subdomains. The GLN domain hydrolyzes glutamine to provide ammonia for carbamoyl phosphate synthesis. Carbamoyl phosphate is synthesized by the concerted action of the homologous CPS.A and CPS.B (35). The ATC domain catalyzes the synthesis of carbamoyl aspartate from carbamoyl phosphate and aspartate. The DHO domain catalyzes the cyclization of carbamoyl aspartate to form dihydroorotate. The allosteric effectors bind to the regulatory subdomain (B3) on the carboxyl end of CPS.B. The MAP kinase site (Thr456), PKA sites, Ser 1406 and Ser 1859, and the autophosphorylation site (Thr1037), as is the putative PKC phosphorylation site (Ser1873), are also shown. *Panel B:* The sequence of the interdomain chain segment connecting the DHO and ATC domains contains the PKA2 and putative PKC phosphorylation sites with the kinase consensus sequences underlined. The phosphorylated residues are in bold type.

2. INTRODUCTION

The demand for pyrimidine nucleotides is greatest during the S phase of the cell cycle to provide precursors for DNA synthesis. The supply of pyrimidines is governed by the activity of CAD, a multifunctional protein that catalyzes the first three steps of the *de novo* pathway (1). The protein is organized into discrete structural domains (Figure 1) that have glutamine-dependent carbamoyl phosphate synthetase (GLN-CPS), aspartate transcarbamoylase (ATC) and dihydroorotase (DHO) activities. The first step in the pathway, the formation of carbamoyl phosphate, which involves the concerted action of the CPSase GLN, CPS.A and CPS.B domains, is the rate limiting step and the major locus of control of *de novo* pyrimidine biosynthesis. The CPSase activity is subject to feedback inhibition by UTP, an end product, and is allosterically activated by PRPP, a substrate for a subsequent step in the pathway and the first step in purine biosynthesis (2-4). The allosteric effectors bind to distinct sites within the regulatory subdomain (B3) at the extreme carboxyl end of the CAD CPS.B domain (5).

Two signaling cascades play an important role in the cell cycle dependent regulation of *de novo* pyrimidine biosynthesis (6). Stimulation of quiescent cells with growth factors such as EGF and PDGF activates the MAP kinase (Erk1/2) cascade which signals entry into the proliferative phase. Just prior to the S phase of the cycle, Thr456 of CAD is phosphorylated by MAP kinase which results in an appreciable

increase in PRPP activation coupled with a loss of UTP inhibition allowing the pyrimidine nucleotide pools to expand. While CAD phosphorylation has no effect on the GLN-CPSase activity, the response to allosteric effectors is modulated resulting in a substantial increase in the flux through the *de novo* pyrimidine biosynthetic pathway. When the cells emerge from the S phase of the cell cycle, CAD Thr456 is dephosphorylated and Ser1406 is phosphorylated by protein kinase A (PKA). Phosphorylation of purified CAD by PKA also abolishes UTP inhibition (7), but appreciably reduces the activation of CAD by PRPP (8). *In vivo*, the combined effect of dephosphorylation of the MAP kinase site and phosphorylation of the PKA site reduces the rate of pyrimidine biosynthesis to the basal level.

Protein kinase C (PKC) also has an important role in proliferation as first discovered, when it was shown to be the target of mitogenic phorbol esters, strong tumor promoters in mouse skin (9). Protein kinase C is a family of at least nine isoforms that differ in mode of activation, tissue distribution (10), intracellular localization (11) and function. Depending on cell type, phorbol esters can have two effects, 1) stimulation of quiescent cells to enter the cell cycle, e.g. 3T3 mouse fibroblasts (12) and resting T lymphocytes (13) and 2) inhibition of cell proliferation and stimulation of differentiation. The latter effect was first demonstrated when the HL60 promyelocytic leukemia cell line was induced to differentiate into a macrophage like phenotype (14) and subsequently the same phenomenon was observed in other cell types (15). Protein kinase C

participates in a complex network of signal transduction pathways involving both tyrosine kinase receptors and G protein coupled receptors. In the canonical MAP kinase pathway, the signal initiated by the binding of growth factors to extracellular receptors is transmitted via Ras, Raf-1 and MEK (MAP kinase kinase) resulting in the phosphorylation and activation of ERK1/2 MAP kinase. Activation of PKC by phorbol esters also leads to stimulation of the MAP kinase cascade by phosphorylating and activating Raf-1, while PKA phosphorylation of a distinct site on Raf-1 can down regulate MAP kinase (16). The central role of PKC in cell growth and division and the identification of a putative PKC phosphorylation site in CAD, reported here, prompted us to investigate the role of PKC on the cell cycle dependent control mechanisms exerted on CAD.

3. MATERIALS AND METHODS

3.1. Cell Culture

BHK 165-23 (17), a baby hamster kidney cell line derived from BHK-21 was grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% dialyzed fetal bovine serum and 2 micrograms/ml gentamicin (Gibco). Two million cells were plated in T75 flasks containing 25 ml of media. The media was changed every two days. Cells were counted using a hemocytometer and viability was assessed by trypan blue staining. Urd'A cells (18) lacking endogenous CAD, used for some of these studies, were grown similarly.

3.2. Preparation of Cell Extracts

The cells were washed twice with ice-cold phosphate buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and harvested in 4 ml ice-cold PBS-PMSF by scraping. The cells were collected and the flasks were rinsed with 4 ml of ice-cold PBS-PMSF. The cell suspension was then centrifuged at $10,000 \times g$ at 4°C for 5 minutes and lysis buffer (200 microliters per 3×10^6 cells, 20 mM TrisHCl, pH 7.5, 137 mM NaCl, 10% glycerol, 1% Triton) supplemented with 1X mammalian protease inhibitor cocktail (Sigma) and 1X mammalian phosphatase inhibitor cocktail (Sigma). The cell suspension was vortexed and sonicated for 10 seconds six times in an ice bath and centrifuged at $24,000 \times g$ at 4°C for 30 minutes. Aliquots of the supernatants were stored at -80°C . Protein quantization was performed by the Lowry method (19) and by scanning stained SDS-polyacrylamide gels calibrated with known amounts of bovine serum albumin as a standard using the software UNSCAN-IT (Silk Scientific Corporation).

3.3. Enzyme Assays

The glutamine-dependent carbamoyl phosphate synthetase (CPSase) assay has been described (20). The 1 ml assay mixture contained 100 micrograms of protein, 100 mM TrisHCl, pH 8.0, 100 mM KCl, 7.5% DMSO, 2.5% glycerol, 1 mM DTT, 3.5 mM glutamine, 20.2 mM aspartate, 1.5 mM ATP, 3.5 mM MgCl_2 and 5 mM sodium [^{14}C] bicarbonate (1.6×10^6 microcuries/mol). The concentration of MgCl_2 was adjusted so as to maintain a 2 mM excess over the sum of the concentration of ATP, UTP

and PRPP. Aspartate transcarbamoylase (ATCase) activity was assayed by the colorimetric method (21). The ATCase assay mixture contained, 100 micrograms of protein, 5 mM carbamoyl phosphate and 12 mM aspartate in a buffer consisting of 100 mM TrisHCl, pH 8.0, 100 mM KCl, 7.5% DMSO, 2.5% glycerol and 1 mM DTT in a total volume of 1 ml. The MAP kinase Erk1/Erk2 activities were assayed by immunoblotting using phospho-ERK1/2 antibodies that recognize only the activated kinases (Promega).

3.4. Immunoblotting

For immunoblotting, the following antibodies were used at the previously described dilution (6, 19, 20, 22) or as recommended by the manufacturer: phosphoserine (Z PS-1, Zymed Laboratories Inc.) rabbit polyclonal antibodies; phospho-threonine (Z PT-1, Zymed Laboratories Inc.) rabbit polyclonal antibodies; diphospho-Erks (anti-Thr202/Tyr204 phosphorylated p42/p44, (Cell Signaling Technology) mouse monoclonal antibody; PKA rabbit polyclonal antibody (Santa Cruz); affinity purified goat anti-mouse IgG (H&L) antibody conjugated to horseradish peroxidase and affinity purified goat anti-rabbit IgG (H&L) antibody conjugated to horseradish peroxidase (Cell Signaling Technology); rabbit polyclonal antibody against all *Aequorea victoria* green fluorescent protein variants (Living Colors A.v., Clontech). The rabbit polyclonal CAD serum was prepared as previously described (23). Immunoblotting and quantization of the immunoblots was carried out as previously described (20).

3.5. Cloning and expression of CAD and GLN CPS

The construct, pECFP-GLN-CPS, encoded CAD GLN-CPS with the enhanced cyan fluorescent protein fused to the amino end (6). For the construction of the full length CAD fusion protein (6), the CAD clone, pCK-CAD10 (24) was cleaved with *XbaI*. The 3750 bp fragment containing the end of the CPS.B domain and the remainder of the CAD coding sequences was inserted into pECFP-GLN-CPS that had been cleaved with *XbaI*. *XbaI* cleaved pECFP-GLN-CPS in the CPS.B domain and in the vector downstream of the insert. The ligation products were transformed into DH5 alpha. The construct, pECFP-CAD, encoded the entire CAD protein with the enhanced cyan fluorescent protein appended to the amino end. The fidelity of all constructs was verified by DNA sequencing (Wayne State University DNA Sequencing Facility).

3.6. Transfection

The *E. coli* transformants were cultured overnight in Luria Broth, 100 micrograms/ml kanamycin and plasmids were purified using the Plasmid Maxi kit (Qiagen). BHK cells were transfected with Lipofectamine Plus reagents (Invitrogen) using 2 micrograms of the DNA following the manufacturer's protocol. After 5 hours, the transfection medium was replaced with DMEM complete medium. The expression of the recombinant proteins was monitored by fluorescence microscopy and Western blotting using antibodies directed against, CAD, MAPK (p44/p42) and GFP/ECFP. The recombinant proteins were fluorescent and had the expected size on calibrated SDS gels.

PKC promotes MAP kinase phosphorylation of CAD

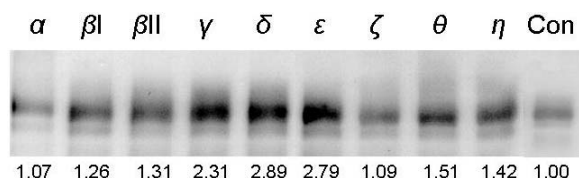


Figure 2. Reaction of purified CAD with PKC isozymes.

Purified CAD (0.33 mg/ml) in 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.1 mM CaCl₂, 10 mM ATP, 500 micro/ml phosphatidyl serine, and 50 micrograms/ml diacylglycerol was reacted with a panel of PKC isozymes in a total volume of 55 microliter for 60 minutes at 30°C. The extent of CAD serine phosphorylation was determined by immunoblotting. The reaction mixture was fractionated by SDS gel electrophoresis, transferred onto nitrocellulose and probed with antibodies directed against phosphorylated serine residues. The relative intensity of the signals was determined by quantitative scanning of the immunoblot as described in the *Experimental Procedures*.

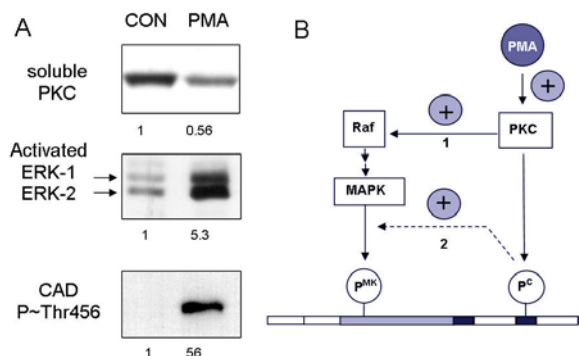


Figure 3. Effect of PKC activators on PKC and ERK activity and the phosphorylation of CAD Thr456. *Panel A:* Confluent cultures of BHK 165-23 cells were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) or with the PMA vehicle, DMSO as a control (CON) for 60 minutes. The cell extracts were analyzed by immunoblotting. The blots were probed with PKC, diphosphorylated Erk 1/2, and CAD phosphor-Thr456 antibodies to determine the soluble PKC, activated MAP kinase, and the extent of phosphorylation of CAD Thr456, respectively. The blots were quantified as described in the *Experimental Methods* and the intensities relative to the control are given under each lane. *Panel B:* Schematic representation of the postulated mechanism of PMA stimulated phosphorylation of CAD Thr456 (P^{MK}). There are two possible pathways that are not mutually exclusive, 1) activation of the MAP kinase cascade by stimulating Raf (16) or 2) direct phosphorylation of CAD (P^C) resulting in increased susceptibility to MAP kinase phosphorylation. The bar represents the CAD polypeptide.

4. RESULTS

4.1. Phosphorylation of CAD by Protein Kinase C

Previous studies have shown that CAD Thr456 (20) and Ser1406 (7) are phosphorylated by MAP kinase

and PKA, respectively and that CAD undergoes autophosphorylation on Thr1037 (22). Mass spectrometry analysis of CAD from extracts of exponentially growing BHK 165-23 cells showed that CAD Ser1873 was also phosphorylated. This residue had been predicted by sequence analysis using the program NetPhos (ExPasy) to be a potential target of protein kinase C. Ser1873, located within the flexible chain segment that connects the ATC and DHO domains of CAD, lies within a sequence, ¹⁸⁷¹KPSRK (Figure 1) that closely matches the PKC consensus sequence, X-S/T-X-R/K.

To determine whether CAD can be phosphorylated by PKC, purified CAD was reacted with MgATP and a panel of PKC isoforms. The incorporation of phosphate into CAD was assessed by immunoblotting with phosphoserine or phosphothreonine specific antibodies. No threonine phosphorylation was detected (not shown). The phosphoserine signal was only slightly above the basal level observed with controls lacking any kinase (Figure 2) for the alpha, beta I, beta II, zeta, theta and eta PKC isoforms indicating that there was minimal CAD phosphorylation catalyzed by these enzymes. However, PKC delta, epsilon and to a lesser extent the gamma isoforms increased the phosphoserine signal 2-3 fold, above background suggesting that these enzymes directly phosphorylate CAD *in vitro*.

4.2. Effect of PKC Activation *in vivo*

Treatment of confluent cells with phorbol 12-myristate 13-acetate (PMA), a selective PKC activator, resulted in a 2-fold decrease in soluble PKC (Figure 3A), an observation consistent with previous studies (25) that showed that the enzyme is translocated to cell membranes upon activation. Exposure to PMA also resulted in a 5-fold activation of ERK 1/2, a phenomenon that has been attributed (26) to the direct PKC mediated phosphorylation and activation of Raf kinase in the MAP kinase cascade. There is little phosphorylation of CAD Thr456 in confluent cells (20) where the rate of pyrimidine biosynthesis is down-regulated. However, exposure of the confluent cultures to PMA resulted in a 56-fold increase in phosphorylation of Thr456 that can probably be attributed in part to the activation of ERK 1/2. However, it is possible that direct phosphorylation of CAD by PKC may also contribute to the enhanced phosphorylation of Thr456 by ERK 1/2. These two activation mechanisms are illustrated schematically in Figure 3B.

4.3. Effect of Kinase Inhibitors on the PMA induced CAD phosphorylation

The increase in CAD Thr456 phosphorylation in confluent BHK 165-23 cells induced by PMA was blocked by the selective PKC inhibitor, bisindolylmaleimide I (27) indicating that the effect on the CAD phosphorylation state was PKC dependent (Figure 4). Similarly, the ERK 1/2 inhibitors, PD98059 and UO126 also abolished the PMA stimulated phosphorylation of Thr456 indicating that optimal phosphorylation depends on both the MAP kinase and PKC. In contrast, the PKA inhibitor H89 resulted in a 50% increase in PMA stimulation of Thr456 phosphorylation or 4-fold over the levels found in control

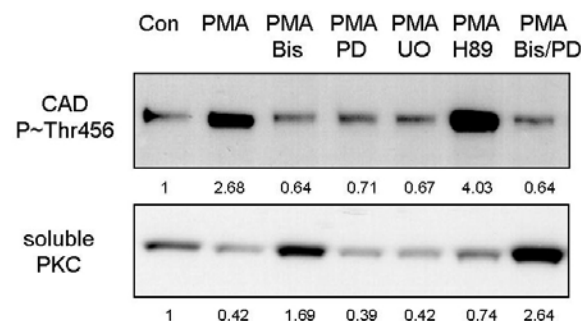


Figure 4. Effect of PKC activators on PKC and ERK activity and the phosphorylation of CAD Thr456. Exponentially growing BHK 165-23 hamster cells were serum starved for 24 hours. The PKC activator, phorbol-12-myristate 13-acetate (PMA), was added to the culture media to a concentration of 100 nM, without inhibitors or in the presence of the PKC inhibitor, bisindolymaleimide I (BIS) at 3 micromolar, or the MEK inhibitors, PD98059 (PD) or UO126 (UO) at 10 micromolar or the protein kinase A inhibitor, H89, at 10 micromolar. The cells were incubated for 10 minutes with the inhibitors prior to the addition of PMA. After stimulation with PMA, the cells were incubated for an additional 60 minutes and the soluble proteins were isolated and fractionated by SDS gel electrophoresis for immunoblotting as described in the *Experimental Procedures*. The blots were probed with phospho-Thr456 or PKC antibodies. Activation of PKC results in its association with the cell membranes and is thus accompanied by a decrease of PKC in the soluble fraction. Control cells (CON) were incubated for the same time periods with comparable volume of the DMSO vehicle without activators or inhibitors. The relative intensity of the signals was determined by quantitative scanning.

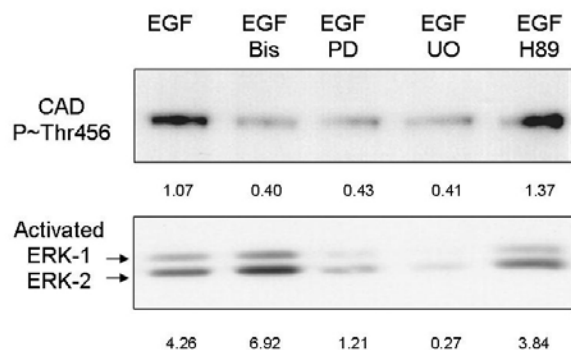


Figure 5. The effect of inhibitors on the EGF stimulation of MAP kinase phosphorylation of CAD. Exponentially growing BHK 165-23 hamster cells were serum starved for 24 hours. The cells were exposed to 4 nM epidermal growth factor (EGF) in the absence or presence of the PKC inhibitor, bisindolymaleimide I (BIS) at 3 micromolar, or the MEK inhibitors, PD98059 (PD) or UO126 (UO) at 10 micromolar or the protein kinase A inhibitor, H89, at 10 micromolar. The soluble proteins were analyzed by immunoblotting. The blots were probed with antibodies directed against phospho-Thr456 or activated MAP kinase Erk1 and Erk2. The relative intensity of the signals was determined by quantitative scanning.

cells. Previous studies have shown (28) that phosphorylation of CAD Ser1406 antagonizes MAP kinase phosphorylation and conversely, that MAP kinase phosphorylation increases when PKA is inhibited. Thus, the extent of Thr456 phosphorylation depends on the interaction of three signaling pathways, MAP kinase, PKC and PKA.

4.4. CAD phosphorylation in EGF stimulated cells

As reported previously (6, 29), exposure of quiescent cells to 5 nM EGF resulted in an appreciable increase in CAD Thr456 phosphorylation. The phosphorylation of this residue correlated well with CAD activation and increased pyrimidine biosynthesis (20). As expected, the MEK inhibitors, PD98059 and UO126 decreased MAP kinase activity 4-16 fold and blocked the EGF induced increase in CAD P~Thr456 (Figure 5). The PKC inhibitor, bisindolymaleimide (Bis), was just as effective in blocking CAD phosphorylation in EGF stimulated cells indicating that CAD phosphorylation proceeds through a PKC-dependent activation of MAP kinase. Thus, both the PKC and MAP kinase cascades participate in the up-regulation of CAD in proliferating cells. In contrast, the PKA inhibitor, H89, slightly increased CAD phosphorylation probably because of the antagonistic interaction of MAP kinase and PKA pathways on CAD phosphorylation (28).

4.5. CAD deletion mutant

Plasmids encoding CAD (CAD) and a CAD deletion mutant (GLN-CPS) lacking the ATC, DHO domains and the interdomain linker containing the putative PKC site were constructed and transfected in UrdA cells that lack endogenous CAD activities. The extent of serine phosphorylation in the quiescent transfectants, was minimal and comparable to the levels found in wild-type mammalian cells (Figure 6A). Stimulation of the transfectants with PMA resulted in an appreciable increase in CAD serine phosphorylation in the cells transfected with the CAD construct. The PMA stimulation of serine phosphorylation was abolished in cells exposed to bisindolymaleimide. In contrast, PMA had no effect on serine phosphorylation in the CPS transfectants in agreement with the tentative location of the PKC phosphorylation site in the interdomain linker. Exposure of the transfectants to EGF resulted in an appreciable increase in Thr456 phosphorylation in CAD transfectants and a significant, although smaller increase in the GLN-CPS transfectants (Figure 6B). As observed with the wild type cells, PMA resulted in an appreciable increase in the CAD transfectants. In contrast, Thr456 phosphorylation of the GLN-CPS mutant was unaffected by PMA suggesting that the PKC target site was missing in the deletion mutant.

5. DISCUSSION

PKC plays a crucial role in cellular proliferation, differentiation and numerous other cellular processes. Its physiological function is dependent on cell type, the PKC isoforms expressed and activity of other signaling networks (30). We report here that CAD, the multifunctional protein that initiates and regulates *de novo* pyrimidine biosynthesis

PKC promotes MAP kinase phosphorylation of CAD

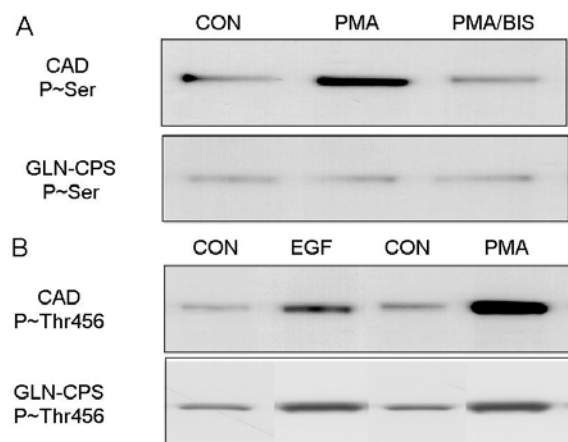


Figure 6. PKC and MAP kinase phosphorylation of CAD in cells transfected with full-length CAD and a truncation mutant. *Panel A:* Urd-A cells were transfected (see *Experimental Procedures*) with constructs encoding full length CAD (CAD) or a deletion mutant in which the ATC and DHO domains (Figure 1) have been excised (GLN-CPS). The transfectants were unstimulated (CON) or stimulated with 100 nM PMA (PMA) or with 5 nM PMA and 3 micromolar bisindolymaleimide I (PMA/BIS). The phosphorylation of serine residues was assessed by immunoblotting. *Panel B:* The Urd-A transfectants described in Panel A were unstimulated (CON) and stimulated with 4 nM EGF or 100 nM PMA. The extent of Thr456 phosphorylation was assessed by immunoblots probed with specific phospho-Thr456 antibodies.

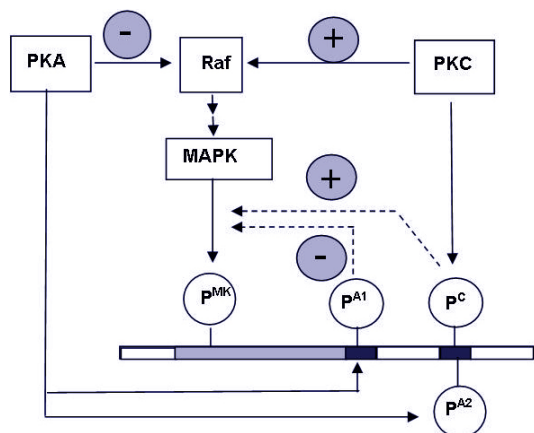


Figure 7. A model for the synergistic and antagonist interactions of the MAP kinase, PKA and PKC signaling pathways on the phosphorylation state of CAD. EGF binding to extracellular receptors triggers the cascade leading to the activation of MAP kinase and the phosphorylation (P^{MK}, Thr456) and activation of CAD. Protein kinase C (PKC) phosphorylates Raf stimulating MAP kinase activity while the protein kinase A (PKA) phosphorylation of Raf is antagonistic. PKA also phosphorylates CAD at two sites (P^{A1}, Ser1406 and P^{A2}, Ser1859). Phosphorylation of P^{A1} decreases the sensitivity to the allosteric activator PRPP, antagonizes the phosphorylation of Thr456 (P^{MK}) that is known to activate CAD and may decrease the phosphorylation of CAD (P^C) by PKC. The phosphorylation of CAD by PKC (P^C) strongly stimulates the phosphorylation of the MAP kinase site (P^{MK}).

in mammalian cells is a PKC target both *in vitro* and *in vivo*. A preliminary screen using a panel of PKC isoforms indicated that while most members of this family of kinases could phosphorylate purified CAD to some extent, the epsilon and delta isoforms were most effective. Different isoforms exhibit a diverse effect on proliferation (30); for example, PKC epsilon is a strong positive regulator, PKC gamma is a weak positive regulator, while PKC delta is considered to be a negative regulator. Recent studies demonstrated that down-regulation of PKC epsilon by RNA interference in quiescent mouse fibroblasts, blocked the activation of the MAP kinase cascade by PMA stimulation (31). These results strongly suggest that the epsilon isoform is essential for PKC activation of MAP kinase. Thus, although this interpretation must be tested experimentally, the up-regulation of PKC epsilon by PMA may be responsible for both the activation of the MAP kinase cascade and the PKC mediated phosphorylation of CAD.

Stimulation of confluent cultures of BHK cells with the phorbol ester, PMA, resulted in a large, 50-fold, increase in the phosphorylation of the Thr456. The phosphorylation of this residue by MAP kinase (Erk1/2) is responsible for the up-regulation of CAD. However, PKC does not directly phosphorylate Thr456 itself since MEK inhibitors that selectively block the MAP kinase cascade were found to also block the effect of PMA on CAD phosphorylation. In principle, two explanations could account for the stimulation of Thr456 phosphorylation (Figure 7); 1) PKC activation of the MAP kinase cascade and 2) direct phosphorylation of CAD by PKC. PKC has been shown to activate MAP kinase by direct phosphorylation and activation of Raf (32) an upstream component of the cascade. Ras, another protein in the signaling pathway has also been shown (31) to be essential for PKC activation of MAP kinase in quiescent mouse fibroblasts. In accord with these studies, PMA stimulation of confluent BHK cells (Figure 3) was found here to activate MAP kinase (Erk 1/2) 5-fold. Secondly, there may be a linkage between MAP kinase and PKC phosphorylation of CAD that promotes phosphorylation of Thr456 when CAD is phosphorylated at a distal site by PKC. At the mechanistic level, this synergistic linkage would be analogous to the antagonistic interaction between the MAP kinase and PKA mediated phosphorylations of CAD (28). In the truncation mutant that has the MAP kinase target sequence but is not phosphorylated by PKC, PMA no longer had any effect on the phosphorylation of Thr456. This result suggests that PMA stimulation of Thr456 phosphorylation is a consequence of direct phosphorylation of CAD by PKC. Both mechanisms are probably operative.

The effect of PMA on Thr456 phosphorylation was also modulated by PKA. Pre-exposure of PMA stimulated cells to the PKA inhibitor, H89, resulted (Figure 4) in a 50% increase in PMA induced phosphorylation of the MAP kinase site. Previous studies (28) have shown that PKA and MAP kinase phosphorylation of CAD are mutually antagonistic. Phosphorylation of CAD Ser1406 by PKA strongly inhibits MAP kinase mediated

PKC promotes MAP kinase phosphorylation of CAD

phosphorylation and vice versa. Thus, inhibition of PKA enhances the stimulation of Thr456 phosphorylation induced by exposure to PKC activators.

In exponentially growing cells, the extent of phosphorylation of Thr456 is appreciably higher than that observed in quiescent cells (20). Nevertheless, phosphorylation of this site increased 2.7 fold upon exposure to PMA. PKC was found to be only partially active in exponentially growing cells. The addition of the PKC inhibitor bisindolymaleimide resulted in a 70% decrease in soluble PKC. Assuming, as a first approximation, all of the PKC was inhibited and released from the membranes, it can be calculated that 30% of the endogenous PKC was active. This interpretation is consistent with the effect of PMA on PKC activity in exponentially growing cells. In the absence of the inhibitor, PMA further activated PKC increasing the membrane bound, active fraction 2.4-fold. This value is close to the 2.7-fold stimulation of Thr456 phosphorylation that occurred upon exposure of exponentially growing cells to PMA.

Similar results were obtained when serum starved cells were stimulated with EGF. In addition to activating the MAP kinase cascade, growth factors binding to tyrosine kinase receptors also have been shown to activate phospholipase C initiating the phosphoinositide cascade that activates PKC (33). Thus, both MAP kinase and PKC signaling pathways are activated when cells enter the proliferative phase. The PKC inhibitor, bisindolymaleimide, did not reduce the MAP kinase activity as observed in confluent cultures presumably because MAP kinase had already been activated by EGF. Although the MAP kinase activity remained elevated, there was a 63% reduction in Thr456 phosphorylation comparable to the inhibition produced by the MEK inhibitors, PD98059 (PD) or UO126 (UO). This effect of bisindolymaleimide reflects the indispensable role that PKC plays in the phosphorylation of Thr456 by MAP kinase. As observed in confluent cultures, inhibition of the antagonistic PKA phosphorylation by H89 resulted in elevated levels of phospho-Thr456.

These results suggest that direct phosphorylation of CAD by PKC promotes phosphorylation of a distal MAP kinase site. While CAD Ser1873 was found to be phosphorylated in exponentially growing cells, the positive identification of this residue as the site of PKC phosphorylation and direct demonstration of a cause and effect relationship between PKC phosphorylation and the stimulation of MAP kinase phosphorylation would be provided by site specific mutants. Preparation of the Ser1873Ala and Ser1873Glu mutants are underway. Two mechanisms can be envisioned that could account for the direct effect of PKC mediated phosphorylation of CAD. In the first, conformational changes induced by PKC phosphorylation may render Thr456 more accessible to MAP kinase. A second attractive hypothesis is that PKC phosphorylation may promote import of CAD into the nucleus where the MAP kinase mediated phosphorylation of Thr456 is known to occur. This interpretation is based

on previous studies (34) showing that a CAD construct incorporating two tandem nuclear export sequences (NES) was confined to the cytoplasmic compartment with the result that the phosphorylation of CAD Thr456 was effectively abolished. It is known however, that the GLN-CPS truncation mutant readily enters the nucleus and is extensively phosphorylated (6) despite the lack of the PKC target site. Perhaps, the smaller size of the deletion mutant facilitates its import without the necessity of further modification.

In summary, several conclusions can be drawn from these results: 1) PKC phosphorylates one or more CAD serine residues *in vitro* and *in vivo*, 2) PKC activation by phorbol esters increases the MAP kinase mediated phosphorylation of Thr456 that activates CAD, 3) the effect of PKC on Thr456 phosphorylation can be attributed in part to an activation of the MAP kinase cascade but also probably to the direct phosphorylation of a different site on CAD by PKC, 4) Activation of CAD by MAP kinase proceeds via a PKC dependent pathway. Thus, CAD is regulated by a complex interplay of three important signaling cascades (Figure 7) that result in mutually antagonist and synergistic phosphorylation of the molecule.

6. ACKNOWLEDGEMENTS

This research was supported by a research grant from the National Institutes of Health, R01-GM60371.

7. REFERENCES

1. D.R. Evans and H.I. Guy: Mammalian pyrimidine biosynthesis: Fresh insights into an ancient pathway. *J. Biol. Chem.* 279, 33035-33038 (2004).
2. S.E. Hager and M.E. Jones: Initial steps in pyrimidine biosynthesis in Ehrlich Ascites Carcinoma *in vitro*. II. The synthesis of carbamoyl phosphate by a soluble, glutamine dependent carbamoyl phosphate synthetase. *J. Biol. Chem.* 242, 5667-5673, (1967).
3. Levine RL, Hoogenraad NJ and Kretchmer N: Regulation of the activity of carbamyl phosphate synthetase from mouse spleen. *Biochemistry* 10, 3694-3699, 1971.
4. M. Tatibana and K Shigesada: Activation by 5-phosphoribosyl 1-pyrophosphate of glutamine-dependent carbamyl phosphate synthetase from mouse spleen. *Biochem. Biophys. Res. Comm.* 46, 491-497 (1972).
5. X. Liu X, H.I. Guy and D.R.Evans: Identification of the regulatory domain of the mammalian multifunctional protein CAD by the construction of an Escherichia coli hamster hybrid carbamyl-phosphate synthetase. *J. Biol. Chem.* 269, 27747-27755 (1994)
6. F. Sigoillot, J. Berkowski, S. Sigoillot, D. Kotsis and H. Guy: Cell cycle dependent regulation of pyrimidine biosynthesis. *J. Biol. Chem.* 278, 3403-3409 (2003)
7. E. A. Carrey, D.G. Campbell and D.G. Hardie: Phosphorylation and activation of hamster carbamyl phosphate synthetase II by cAMP-dependent protein kinase. A novel mechanism for regulation of pyrimidine nucleotide biosynthesis. *Embo. J.* 4, 3735-42, 1985.
8. N. Sahay, H.I. Guy, X. Liu and D.R. Evans: Regulation of an Escherichia coli/mammalian chimeric carbamoyl-

- phosphate synthetase. *J. Biol. Chem.* 273, 31195-202 (1998)
9. A. Viaje, T.J. Slaga, M. Wigler and I.B. Weinstein: Effects of antiinflammatory agents on mouse skin tumor promotion, epidermal DNA synthesis, phorbol ester-induced cellular proliferation, and production of plasminogen activator. *Cancer. Res.* 37, 1530-1536 (1977)
10. W.C. Wetsel, W.A. Khan, I. Merchenthaler, H. Rivera, A.E. Halpern, H.M. Phung, A. Negro-Vilar, and Y.A. Hannun: Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. *J. Cell. Biol.* 117, 121-33 (1992)
11. A.T. Sim and J.D. Scott: Targeting of PKA, PKC and protein phosphatases to cellular microdomains. *Cell. Calcium* 26, 209-17 (1999)
12. E. Rozengurt: Early signals in the mitogenic response. *Science* 234: 161-6 (1986).
13. N. Berry and Nishizuka Y: Protein kinase C and T cell activation. *Eur. J. Biochem.* 189, 205-14, 1990.
14. G.R. Vandenbark, L.J. Kuhn and J.E. Niedel: Possible mechanism of phorbol diester-induced maturation of human promyelocytic leukemia cells. *J. Clin. Invest.* 73, 448-57 (1984)
15. Y. Yamauchi, K. Nagasawa, T. Mayumi, T. Horiuchi and Y. Niho: Activation of protein kinase C induces differentiation in the human T-lymphoblastic cell line MOLT-3. *Br. J. Cancer* 60, 15-19 (1989)
16. E. Maioli, C. Torricelli and V. Fortino: Functional interactions of protein kinase A and C in signaling networks: a recapitulation. *Cell. Mol. Life Sci.* 63, 637-641 (2006)
17. T. Kempe, E. Swyryd, M. Bruist and G. Stark: Stable mutants of mammalian cells that overproduce the first three enzymes of pyrimidine synthesis. *Cell* 9, 541-550 (1976)
18. D. Patterson and D. Carrington: Biochemical genetic analysis of pyrimidine biosynthesis in mammalian cells: I. Isolation of a mutant defective in the early steps of de novo pyrimidine biosynthesis. *Somatic Cell Genet.* 5, 483-495 (1977)
19. O. Lowry, N. Rosenbrough, A. Farr and R. Randall: Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.* 193, 265-275 (1951)
20. F.D. Sigoillot, D.R. Evans and H.I. Guy: Growth-dependent regulation of mammalian pyrimidine biosynthesis by the protein kinase A and MAPK signaling cascades. *J. Biol. Chem.* 277, 15745-15751 (2002).
21. S.C. Pastra-Landis, J. Foote J and E.R. Kantrowitz: An improved colorimetric assay for aspartate and ornithine transcarbamylases. *Anal. Biochem.* 118, 358-363 (1981)
22. F.D. Sigoillot, D.R. Evans and H.I. Guy HI: Autophosphorylation of the Mammalian multifunctional protein that initiates de novo pyrimidine biosynthesis. *J. Biol. Chem.* 277: 24809-24817 (2002)
23. D.R. Grayson, L. Lee and D.R. Evans: Immunochemical analysis of the domain structure of CAD, the multifunctional protein that initiates pyrimidine biosynthesis in mammalian cells. *J. Biol. Chem.* 260, 15840-15849 (1985)
24. H.I. Guy and D.R. Evans: Cloning and expression of the mammalian multifunctional protein CAD in *Escherichia coli*. Characterization of the recombinant protein and a deletion mutant lacking the major interdomain linker. *J. Biol. Chem.* 269, 23808-23816 (1994)
25. A.S. Kraft and W.B. Anderson: Phorbol esters increase the amount of Ca²⁺, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* 301, 621-623 (1983)
26. P.H. Sugden and A. Clerk: Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. *Cell Signal.* 9, 337-351 (1997)
27. G. Martiny-Baron, M.G. Kazanietz, H. Mischak, P.M. Blumberg, G. Kochs, H. Hug, D. Marme and C. Schachtele: Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J. Biol. Chem.* 268, 9194-9197 (1993)
28. D.H. Kotsis, E.M. Masko, F.D. Sigoillot, R.D. Gregorio, H.I. Guy-Evans and D.R. Evans: Protein kinase A phosphorylation of the multifunctional protein CAD antagonizes activation by the MAP kinase cascade. *Mol. Cell. Biochem.* Jan 6 0300-8177 (Print), (2007)
29. L.M. Graves, H.I. Guy, P. Kozlowski, M. Huang, E. Lazarowski, R.M. Pope, M.A. Collins, E.N. Dahlstrand, H.S. Earp III and Evans DR: Regulation of Carbamoyl Phosphate Synthetase by MAP kinase. *Nature* 403: 328-332, 2000.
30. M.J. Clemens, I. Trayner and J. Menaya: The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. *J. Cell. Sci.* 103 (Pt 4), 881-887 (1992)
31. M. Hamilton, J. Liao, M.K. Cathcart and A. Wolfman: Constitutive association of c-N-Ras with c-Raf-1 and protein kinase C epsilon in latent signaling modules. *J. Biol. Chem.* 276, 29079-29090 (2001)
32. W. Kolch, G. Heidecker, G. Kochs, R. Hummel, H. Vahidi, H. Mischak, G. Finkenzeller, D. Marme and U.R. Rapp: Protein kinase C alpha activates RAF-1 by direct phosphorylation. *Nature* 364, 249-252 (1993)
33. A.B. da Rocha, D.R. Mans, A. Regner and G. Schwartsmann: Targeting protein kinase C: new therapeutic opportunities against high-grade malignant gliomas? *Oncologist* 7, 17-33 (2002)
34. F.D. Sigoillot, D.H. Kotsis, V. Serre, S.M. Sigoillot, D.R. Evans and H.I. Guy: Nuclear localization and mitogen-activated protein kinase phosphorylation of the multifunctional protein CAD. *J. Biol. Chem.* 280, 25611-25620 (2005)
35. A. Meister: Mechanism and regulation of the glutamine-dependent carbamyl phosphate synthetase of *Escherichia coli*. *Adv. Enzymol. Relat. Areas Mol. Biol.* 62, 315-374 (1989)

Key Words: CAD, pyrimidine biosynthesis, signaling cascades, MAP kinase, Erk1/2, protein kinase A, protein kinase C

Send correspondence to: Hedeel Guy Evans Ph.D., Department of Chemistry, Eastern Michigan University, Ypsilanti, Michigan, 48197, Tel: 734-487-0106, Fax: 734-487-1496, E-mail: hguy@genetics.wayne.edu

<http://www.bioscience.org/current/vol12.htm>