Growth-dependent Regulation of Mammalian Pyrimidine Biosynthesis by the Protein Kinase A and MAPK Signaling Cascades

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The carbamoyl phosphate synthetase domain of the multifunctional protein CAD catalyzes the initial, rate-limiting step in mammalian de novo pyrimidine biosynthesis. In addition to allosteric regulation by the inhibitor UTP and the activator PRPP, the carbamoyl phosphate synthetase activity is controlled by mitogen-activated protein kinase (MAPK)- and protein kinase A (PKA)-mediated phosphorylation. MAPK phosphorylation, both in vivo and in vitro, increases sensitivity to PRPP and decreases sensitivity to the inhibitor UTP, whereas PKA phosphorylation reduces the response to both allosteric effectors. To elucidate the factors responsible for growth state-dependent regulation of pyrimidine biosynthesis, the activity of the de novo pyrimidine pathway, the MAPK and PKA activities, the phosphorylation state, and the allosteric regulation of CAD were measured as a function of growth state. As cells entered the exponential growth phase, there was an 8-fold increase in pyrimidine biosynthesis that was accompanied by a 40-fold increase in MAPK activity and a 4-fold increase in CAD threonine phosphorylation. PRPP activation increased to 21-fold, and UTP became a modest activator. These changes were reversed when the cultures approach confluence and growth ceases. Moreover, CAD phosphoserine, a measure of PKA phosphorylation, increased 2-fold in confluent cells. These results are consistent with the activation of CAD by MAPK during periods of rapid growth and its down-regulation in confluent cells associated with decreased MAPK phosphorylation and a concomitant increase in PKA phosphorylation. A scheme is proposed that could account for growth-dependent regulation of pyrimidine biosynthesis based on the sequential action of MAPK and PKA on the carbamoyl phosphate synthetase activity of CAD.

The rate of de novo pyrimidine biosynthesis parallels the growth rate of the cell, and there is good evidence (1–13) that the activation of the pathway is necessary for proliferation of tumor and neoplastic cells. In mammalian cells, the pathway consists of six steps (Fig. 1A) that result in the formation of UMP. The flux of metabolites through the pathway (14) is controlled by carbamoyl phosphate synthetase (CPSase), the enzyme that catalyzes the first committed and rate-limiting step of the pathway. Mammalian CPSase is part of a large multifunctional protein called CAD (15–17) that also carries aspartate transcarbamoylase (ATCase) and dihydroorotase (DHOase) activities, enzymes that catalyze the second and third steps of the pathway, respectively. The 243-kDa CAD polypeptide (Fig. 1B) is organized (18–20) into multiple domains, subdomains, and linkers, each with a specific function. The CPSase activity of CAD is allosterically regulated by the inhibitor UTP and the activator PRPP (13, 21–26). A domain swapping experiment (27) clearly showed that the allosteric ligands bind to a regulatory subdomain (B3) at the extreme carboxyl end of the CPS.B domain of CAD (Fig. 1B).

Carrey and co-workers (26, 28, 29) discovered that purified CAD is phosphorylated by cAMP-dependent protein kinase A (PKA). Phosphorylation does not alter the catalytic activity of CPSase or any of the other CAD activities but results in the loss of sensitivity to the allosteric inhibitor, UTP. There are two PKA phosphorylation sites, one located within the B3 regulatory subdomain and a second in the interdomain linker that connects the ATC and DHO domains (Fig. 1B). Desensitization to UTP correlates with the phosphorylation of Ser<sup>1406</sup> in the regulatory subdomain. Banerjei and Davidson (30) showed that in transfected cells, replacement of Ser<sup>1406</sup> with glutamate mimics the effects of in vitro phosphorylation of CAD. The phosphorylation of Ser<sup>1406</sup> in the interdomain linker has no effect on allosteric regulation but has been implicated in carbamoyl phosphate channeling (31). PKA-mediated phosphorylation was considered an activation mechanism that could explain growth-dependent changes in pyrimidine biosynthesis in vivo because the constraints imposed on the pathway by UTP inhibition are effectively abolished.

More recently, it was discovered (32) that the response to PRPP is also diminished by PKA phosphorylation. The maximum allosteric activation is unchanged, but the affinity of PRPP for CPSase is substantially weakened. Thus, depending on the intracellular concentration of allosteric ligands, PKA-mediated phosphorylation might be expected to either up- or down-regulate the activity of the pathway. Although cAMP is required for the growth of yeast, in most mammalian cells (about 75% of the cases examined), it is considered (33) antagonistic to cell growth and proliferation. Therefore, cAMP may

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The abbreviations used are: CPSase, carbamoyl phosphate synthetase; ATCase, aspartate transcarbamoylase; DHOase, dihydroorotase; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PALA, N-phosphonoacetyl-L-aspartate; PKA, protein kinase A; GST, glutathione S-transferase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; EGF, epidermal growth factor.
serve in many instances as a counter-regulator to signals generated by insulin and growth factors. Thus, there are likely to be other mechanisms, in addition to PKA phosphorylation, that override the allosteric constraints on CAD activity when cells enter the proliferative phase.

Recently, CAD was found (34) to be regulated both in vivo and in vitro by the MAPK cascade. MAPKs (35), such as extracellular signal-regulated kinases (Erks) 1 and 2, are ubiquitous components of the mitogen-activated cascade that result in cellular proliferation in response to growth factors. Erk1 and Erk2 are also activated by oncogene products (36). MAPK phosphorylates a Thr456 in the A1 subdomain of the CAD CPS (Fig. 1B). Although far from the regulatory domain in the linear sequence, molecular modeling based on the known structure of *Escherichia coli* CPSase (37) suggests that it lies in close proximity to the B3 regulatory subdomain. MAPK-mediated phosphorylation, like that of PKA, abolishes UTP inhibition; however, PRPP activation is markedly stimulated. Both the loss of sensitivity to UTP and increased sensitivity to PRPP would be expected to activate CPSase and are thus likely to be important for regulation of pyrimidine biosynthesis.

These observations suggest that mammalian CPSase is likely to be controlled in vivo by the interplay of allosteric effectors and two different signaling cascades. Here, we show that activation of pyrimidine biosynthesis in rapidly growing cells is associated with phosphorylation by MAPK and that PKA phosphorylation is involved in its down-regulation as cell growth is arrested by contact inhibition.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—BHK 165-23 (38) is a baby hamster kidney cell line derived from BHK-21 in which the CAD gene was amplified by exposure to the ATCase inhibitor N-phosphonacetyl-L-aspartate (PALA). The cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% dialyzed fetal bovine serum and 2 μg/ml gentamicin (Invitrogen). Two million cells were plated in 75-cm² flasks containing 25 ml of media. The media were changed every 2 days. The cells were judged to be confluent when nearly all of the attached cells were in contact with one another, and cell growth had ceased. Cells were counted using a hemocytometer, and viability was assessed by trypan blue staining. Although CAD is overexpressed in these cells, the growth-dependent variation in the rate of pyrimidine biosynthesis is identical to that observed in other cell types (39–41), and the elevated levels of protein greatly facilitate quantitation of the phosphorylation state.

**Pyrimidine Biosynthesis Assay**—The rate of de novo pyrimidine biosynthesis was measured as described by Huisman et al. (1) and Fairbanks et al. (42). A suspension of cells (0.16–1.6 × 10⁶) in 90 μl of Dulbecco’s modified Eagle’s medium/F-12 without bicarbonate was placed in a 1-ml microfuge tube. A parallel assay mixture was prepared

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**Fig. 1.** *De novo* pyrimidine biosynthetic pathway and the domain structure of CAD. A, the first three steps in the *de novo* pyrimidine biosynthetic pathway are catalyzed by (1) CPSase, (2) ATCase, and (3) DHOase activities carried by the multifunctional protein CAD. The CPSase activity controls the flux through the entire six-step pathway. B, the domain structure of the 243-kDa CAD polypeptide. The synthesis of carbamoyl phosphate is catalyzed by the concerted action of the glutaminase (GLN), CPS.A, and CPS.B domains. The allosteric ligands, UTP and PRPP, bind to the B3 regulatory domain of CPS.B. The CPSase activity is also controlled by phosphorylation of the MAPK and PKA1 sites. There is a second PKA target site (PKA2) unrelated to regulation. The formation of carbamoyl aspartate and dihydroorotate is catalyzed by the ATC and DHO domains, respectively.

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[2] A. Hewagama, T. Lu, L. Kovari, H. I. Guy, and D. R. Evans, unpublished observations.
that was identical, except that it contained 1 mM PALA (a gift of Drs. V. Narayanan and I. Reddi of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NIH, Silver Spring, MD), a potent bisubstrate inhibitor of ATCase that completely blocks (43) the flux through the de novo pyrimidine biosynthetic pathway. The reaction was initiated by the addition of 10 μl of 9.1 mM sodium [14C]bicarbonate (220 μCi/μmol; ICN Inc.). The cell suspension was incubated at 37 °C for 5, 10, 20, 30, 45, and 60 min and then pelleted by centrifugation at 10,000 × g for 1 min in a microfuge. The cells were washed twice in ice-cold phosphate-buffered saline and then lysed by the addition of 200 μl of 10% trichloroacetic acid. The trichloroacetic acid extract was pelleted by centrifugation at 12,000 × g for 1 min. The supernatant was counted, and in some instances, the trichloroacetic acid was removed by back extraction with water-saturated diethyl ether, pH 5.0, for high pressure liquid chromatography analysis. The incorporation of [14C]bicarbonate into RNA and DNA was measured by counting the trichloroacetic acid pellets after dissolving them in 200 μl of 0.1 N NaOH. The progress curves were linear over a 60-min period. The rate of de novo pyrimidine biosynthesis was taken as the difference between the rate of bicarbonate incorporation into acid-soluble metabolites in the presence and absence of PALA.

Preparation of Cell Extracts—The cells were washed twice with ice-cold phosphate-buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride and harvested in 4 ml of ice-cold phosphate-buffered saline-phenylmethylsulfonyl fluoride by scraping. Cells were collected, and the flasks were rinsed with 4 ml of ice-cold phosphate-buffered saline-phenylmethylsulfonyl fluoride by scraping. The cell suspension was centrifuged at 10,000 × g at 4 °C for 5 min, and a lysis buffer consisting of 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol, and 1% Triton supplemented with mammalian protease inhibitor mixture and mammalian phosphatase inhibitor mixture (Sigma) was added (200 μl of 106 cells). The cell suspension was vortexed to dissociate cell clumps, sonicated for 1 min in a microfuge. The cells were washed twice in ice-cold phosphate-buffered saline and then lysed by the addition of 10% trichloroacetic acid. The trichloroacetic acid extract was pelleted by centrifugation at 12,000 × g for 1 min. The supernatant was counted, and aliquots were stored at −80 °C. In some instances, CAD was isolated by affinity chromatography on a PALA column (44), and its identity was verified by immunoblotting using CAD-specific antibodies. Protein quantitation was performed by using the method of Lowry et al. (45).

Antibodies—The phosphoserin (Z PS-1) and phosphothreonine (Z PT-1) rabbit polyclonal antibodies against ZH11002 (Amersham Biosciences) were used for immunoblotting at a dilution of 1:5000. The anti-CAD antibodies were obtained from Cell Signaling Technology.

Immunoblotting—SDS-PAGE on 5% or 10% gels was carried out as described previously (48). The Coomassie blue-stained gels were dried, and protein bands were visualized by autoradiography on Kodak XAR-5 film (308C). Signals were visualized using Biomax ML film (Kodak) and quantitated by scanning the immunoblots with a Hewlett-Packard ScanJet 4c and UNSCAN-IT software (Silk Scientific Corp.). Care was taken to ensure that all exposures fell within the linear response range of the film. Concentrations of proteins in each sample were determined by using the Bradford reagent (Bio-Rad Laboratories) and the kit instructions. The antigen concentrations in the immunoprecipitates were determined by using the dilution factor of the sample.

ATCase and DHOase Assays—The ATCase and DHOase activities were determined using the previously described colorimetric method (49, 50). The ATCase assay mixture contained 5 mM carbamoyl phosphate and 12 mM aspartate in a buffer consisting of 100 mM Tris-Cl, 100 mM KCl, 7.5% Me2SO, 2.5% glycerol, adjusted at pH 8.0, 1 mM dithiothreitol, 3.5 mM glutamine, 20.2 μM aspartate, 1.5 mM ATP, 3.5 mM MgCl2, and 5 mM sodium [14C]bicarbonate (1.6 × 106 μCi/mol). When UTP and/or PRPP were included, the concentration of MgCl2 was adjusted to maintain a 2-fold excess over the sum of the concentration of ATP, UTP, and PRPP. The reaction was performed at 37 °C for 15 min, quenched by the addition of 1 ml of 40% trichloroacetic acid, and heated at 100 °C for 15 min. Approximately 0.2 g of dry ice was added to the vials to eliminate the excess CO2 generated during the reaction, and then the vials were heated at 100 °C for an additional 15 min before counting in a Beckman-Coulter counter.

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cAMP-dependent PKA Assays—The PKA activity in the cell extract (20–150 μg of protein) was determined using the PKA assay kit (Invitrogen) following the manufacturer’s recommendations. The assay is based on the phosphorylation of the specific PKA substrate kemptide by

FIG. 3. Growth-dependent phosphorylation of CAD. A, cells were harvested on the indicated days, and the relative phosphorylation of CAD was determined by immunoblotting using antibodies directed specifically against phosphothreonine. Enzyme assays and immunoblotting with anti-CAD antibodies showed that the concentration of CAD remained constant throughout the growth curve. B, the phosphorylation of CAD on threonine (●) and serine (○) residues in cells harvested at the indicated growth stage was determined by scanning the immunoblot as described under “ Experimental Procedures.”
Regulation of Mammalian Pyrimidine Biosynthesis

**Growth Phase Dependence of Pyrimidine Biosynthesis**—Growth curves were obtained by seeding BHK 165-23 cells at a concentration of \(2 \times 10^5\) cells/25 ml in 75-cm\(^2\) T flasks and monitoring cell number (Fig. 2) over a period of 5–6 days. After an initial lag period, the cells entered an exponential growth phase during which the cell number increased to \(17 \times 10^6\) over a period of 4 days. As the cells became confluent, there was strong contact inhibition that arrested growth. Thereafter, the cell number began to gradually decline.

The rate of pyrimidine biosynthesis was assayed by measuring the time-dependent incorporation of \(^{14}\)C]bicarbonate into nucleotides and pathway intermediates in the presence and absence of 1 mM N-phosphonacetyl-L-aspartate, a highly specific bisubstrate ATCase inhibitor that blocks entry into the pyrimidine biosynthetic pathway. The specific flux through the pyrimidine pathway corresponds to the difference in the rate of total incorporation and the incorporation in the presence of PALA. Typically, pyrimidine biosynthesis corresponds to 60% of the total incorporation. Incorporation proceeded linearly for at least 1 h.

In resting cells, the rate of pyrimidine biosynthesis was found to be 0.5 pmol/min/10^6 cells. During exponential growth, the rate increased to 4 pmol/min/10^6 cells and then dropped precipitously to the basal level when the cells entered the confluent growth phase. Immunoblots using CAD-specific antibodies as well as CAD isolation by affinity chromatography on PALA-Sepharose columns (data not shown) demonstrated that the intracellular concentration of CAD remained constant throughout the growth curve. These results demonstrate that the activity of the de novo pyrimidine biosynthetic pathway closely mirrors the growth rate of the cells.

**Phosphorylation of CAD**—As described above, the allosteric response of CAD CPYPSase activity is regulated by phosphorylation of Ser\(^{1406}\) by PKA and phosphorylation of Thr\(^{456}\) by MAPK. Consequently, growth state-dependent CAD phosphorylation was monitored using antibodies directed specifically against phosphoserine and phosphothreonine that could discriminate between these two phosphorylation states. These antibodies have been used to quantitate the phosphorylation of purified CAD by the isolated MAPK and PKA (data not shown). The intensity of the signals on the immunoblots correlated well with the extent of incorporation of [\(^{\gamma}\)P]ATP into purified CAD catalyzed by the two kinases.

Immunoblots of CAD from cells harvested in the early stages of cell growth showed that the level of phosphothreonine was low (Fig. 3A) but increased appreciably during exponential growth and then declined abruptly to the basal level when the cells became confluent. Changes in the extent of serine and threonine phosphorylation were quantitated by scanning the immunoblots (Fig. 3B). Whereas CAD threonine phosphorylation increased 2.5-fold during the exponential phase, the level of serine phosphorylation remained low and began to increase only toward the end of the exponential growth phase. Serine phosphorylation reached a maximum increase of 2.5-fold in fully confluent cells and then began to decline. These results are consistent with the interpretation that phosphorylation of CAD by MAPK is highest during exponential growth and declines when growth is arrested, whereas the level of CAD phosphorylation by PKA is low during the exponential growth phase and subsequently peaks when the cells become confluent.

**Growth-dependent Changes in Kinase Activity**—The activity of MAPK, measured by the specific phosphorylation of the substrate Elk1, was very low in newly seeded cells and in the early exponential growth phase (Fig. 4) but increased rapidly as the cells began to grow. MAPK activity peaked in the mid-exponential cells and then began to decline as the cells approached confluence. The relative MAPK activity in the mid-exponential phase cells was 40-fold higher than the values observed in resting or confluent cells. In contrast, the activity of PKA (Fig. 4), assayed by phosphate incorporation into the specific PKA kemptide substrate, increased only slightly as a function of growth state.

**Growth-dependent Changes in Allosteric Regulation**—The growth-dependent changes in the rate of pyrimidine biosynthe-
Pulse-chase experiments have shown that the phosphorylation of Thr456 by MAPK is rate limiting for the production of the de novo pyrimidine biosynthesis. However, the actual rate of flux through the de novo pyrimidine biosynthetic pathway was not measured. Moreover, the presence of UTP at concentrations of up to 1 mM, suggesting that the nucleotide cannot effectively counter the increased PRPP activation that develops in rapidly growing cultures.

A more detailed analysis of CPSase sensitivity to allosteric effectors was obtained by measuring the response of isolated CAD to increasing concentrations of PRPP (Fig. 5). The maximum PRPP activation of CAD from newly seeded cells was 10-fold. However, the extent of activation increased as growth continued, reaching a maximum of 22-fold in mid-exponential cells. As the cells became confluent, there was a gradual decline in PRPP activation of CAD. By day 6, when the cells were fully confluent, the maximum activation of CPSase was only 6-fold.

In contrast, the response to UTP was rather small (Table I), so that the predominant growth state-dependent changes in CAD regulation are associated with changes in sensitivity to the allosteric activator, PRPP. The relative insensitivity of CPSase to pyrimidine nucleotides may have important consequences in the cell because increases in the intracellular pool of UTP, such as the 2-fold increase observed in EGF-stimulated cells (34), would not appreciably reduce the rate of pyrimidine biosynthesis. UTP inhibition and PRPP activation of purified CAD are antagonistic (32, 51) in the sense that UTP reduces PRPP activation and vice versa. Similar results were obtained for CAD isolated from mid-exponential cells (Fig. 6). However, it is significant that PRPP strongly activated CPSase even in the presence of UTP at concentrations of up to 1 mM, suggesting that the nucleotide cannot effectively counter the increased PRPP activation that develops in rapidly growing cultures.

**DISCUSSION**

Previous studies (34) showed that CAD isolated from EGF-stimulated cells exhibited changes in allosteric regulation that would be expected to promote de novo pyrimidine biosynthesis. The formation of carbamoyl phosphate by the CPSase component as growth proceeded. The extent of activation by 50 μM PRPP increased during exponential growth and then returned to the basal level when cells became confluent. Similarly, 1 mM UTP inhibited CPSase activity in early exponential and, to some extent, confluent cells but was a modest activator in rapidly growing cells.

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**FIG. 5.** Growth state-dependent changes in the allosteric regulation of CAD. The CPSase activity in the absence of allosteric effectors remained unchanged throughout the growth curve, but there were appreciable changes in the response to PRPP. The PRPP response curve of CAD was measured in cells harvested immediately after passage (day 1, △) and in early exponential (day 2, ▲), exponential (day 3, ■; day 4, ◆), and confluent cells (day 5, ○; day 6, □).

**FIG. 6.** Effect of PRPP on UTP inhibition in CAD from exponentially growing cells. The UTP response curve of CAD from exponentially growing cells (day 4) was measured in the absence of PRPP (□) and in the presence of 5 μM (●), 20 μM (○), and 50 μM (●) PRPP.

Cell growth by contact inhibition are unknown. In this report, we show that the flux through the de novo pyrimidine pathway increases 8-fold when resting cells enter the exponential growth phase and then decreases to basal levels when the cells become confluent and growth ceases.

PKA phosphorylates two serine residues of CAD (Ser^{1406} and
whereas MAPK phosphorylates a specific threonine residue (Thr^{456}). Consequently, the phosphorylation state could be measured using specific antibodies directed against phosphothreonine and phosphoserine. We cannot rule out with certainty the participation of additional kinases because the size and complexity of the CAD molecule complicate the direct determination of all phosphorylation sites. However, we are confident for several reasons that the observed changes in phosphorylation are due, at least in large part, to MAPK and PKA: (a) the maximum CAD threonine phosphorylation was observed in rapidly growing, mid-exponential cells, corresponding to the peak MAPK activity in the cell, (b) the maximum CAD threonine phosphorylation by MAPK occurs when the cells begin to grow exponentially after EGF stimulation (34), (c) MEK inhibitor PD98059 and PKA inhibitor H89 partially reversed threonine and serine phosphorylation, respectively (data not shown), (d) the allosteric response to PRPP and UTP of CAD with high threonine phosphorylation was identical to the results obtained after MAPK phosphorylation of CAD both in vivo and in vitro (34), and (e) when CAD phosphoserine was elevated, the response to PRPP was reduced, a response similar to that observed when CAD is phosphorylated by PKA in vitro. Thus, the growth-dependent changes in the CAD phosphorylation state are likely to be mediated by MAPK and PKA.

The observed changes in the allosteric response to UTP and PRPP are similar to the results obtained with EGF-stimulated cells (34) and with purified CAD upon phosphorylation with MAPK and PKA (28, 32). In exponentially growing cells, CAD was activated 11-fold by 50 μM PRPP compared with 3.2-fold for cells in the early exponential growth phase (Table I). UTP activated the CPSase from exponentially growing cells by 119% but was an inhibitor of CAD isolated from early exponential cells (58%). When the cells became confluent, PRPP activation decreased; there was only a 2-fold activation by 50 μM PRPP, which is less than the activation observed in early exponential cells, a result consistent with the diminished sensitivity of CPSase to the activator after PKA phosphorylation. UTP, although no longer an activator, did not inhibit the CPSase activity of confluent cells to any great extent (14%), which was as expected because PKA-mediated phosphorylation, like MAPK-mediated phosphorylation, diminishes inhibition by the nucleotide. Therefore, the dominant factor responsible for regulating the CPSase activity in vivo appears to be the response of the enzyme to PRPP.

Depending on the intracellular concentration of the effectors, the growth-dependent modulation of the allosteric response could fully account for the observed changes in the rate of de novo pyrimidine biosynthesis. For example, in the presence of 50 μM PRPP and 1 mM UTP, the CPSase activity from cells harvested in the early exponential growth stage (day 1) was 0.62 nmol/min/mg, compared with a value of 3.78 nmol/min/mg for CAD from exponentially growing cultures (day 4). This 6-fold increase in CAD CPSase activity would be expected to result in a proportional increase in the rate of de novo pyrimidine biosynthesis, close to the experimentally observed value. Experiments are under way to determine the growth-dependent changes in the intracellular concentration of UTP and PRPP.

A model depicting the growth-dependent regulation of CAD CPSase and the de novo pyrimidine biosynthetic pathway is shown schematically in Fig. 7. In newly seeded cells, the level of phosphorylation of CAD CPSase is low, as is the flux through the pyrimidine pathway. As the culture progresses to the exponential phase, MAPK is activated, Thr^{456} of CAD is phosphorylated, and CPSase is activated. This modification leads to an appreciable increase in pyrimidine biosynthesis. Ser^{1406} remains unphosphorylated because CAD-Thr^{456}–P is a relatively poor substrate for PKA. Once the culture enters the confluent stage, MAPK is deactivated, Thr^{456} is dephosphorylated, and Ser^{1406} is phosphorylated by PKA. The combination of phosphorylation of the PKA site and dephosphorylation of the MAPK site leads to a decrease in PRPP sensitivity, lower CPSase activity, and a down-regulation of pyrimidine biosynthesis. Many aspects of this model remain to be tested. Nevertheless, it is clear that the CPSase activity of CAD and the flux through the de novo pyrimidine biosynthetic pathway is governed by the sequential action of two central signaling cascades.

Acknowledgments—We thank Drs. V. Narayanan and L. Kedda for the generous gift of PALA.

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J. Biol. Chem. 2002, 277:15745-15751.
doi: 10.1074/jbc.M201112200 originally published online February 28, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201112200

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