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Review

De novo synthesis of pyrimidine nucleotides; emerging interfaces with signal transduction pathways

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Abstract. The de novo biosynthesis of pyrimidine nucleotides provides essential precursors for multiple growth-related events in higher eukaryotes. Assembled from ATP, bicarbonate and glutamine, the uracil and cytosine nucleotides are fuel for the synthesis of RNA, DNA, phospholipids, UDP sugars and glycogen. Over the past 2 decades considerable progress has been made in elucidating the mechanisms by which cellular pyrimidines are modulated to meet the needs of the cell. Recent studies demonstrate that CAD, a rate-limiting enzyme in the de novo synthesis of pyrimidines, is regulated through reversible phosphorylation, Myc-dependent transcriptional changes and caspase-mediated degradation. These studies point to increasing evidence for cooperation between key cell signaling pathways and basic elements of cellular metabolism, and suggest that these events have the potential to determine distinct cellular fates, including growth, differentiation and death. This review highlights some of the recent advances in the regulation of pyrimidine synthesis by growth-factor-stimulated signaling pathways.

Key words. Phosphorylation; carbamoyl phosphate synthetase; dihydroorotase; CTP synthetase; MAPK; PKA; myc.

Introduction

Years of intensive research have been rewarded with a wealth of knowledge about the basic pathways that determine pyrimidine nucleotide metabolism in mammalian cells. The realization that the activities of many of the pyrimidine synthetic enzymes were elevated in tumor cells suggested that these enzymes could provide fruitful targets for antineoplastic agents [1]. Considerable research has been devoted to designing selective inhibitors of pyrimidine synthesis and has resulted in some of the most successful drugs for the treatment of leukemia and other types of cancer. Moreover, these studies have contributed extensively to our understanding of the enzymes in the pyrimidine biosynthetic pathways, the cellular requirements for these nucleotides in both normal and cancerous cells and the mechanisms of drug resistance that limit the clinical application of pyrimidine synthesis inhibitors [2].

Over the past decade, extensive advances have been made in elucidating the mechanisms by which signals are transmitted from cell surface receptors to intracellular targets [3, 4]. Receptor-mediated signal transduction involves reversible protein phosphorylation that culminates in changes in transcriptional, translational and cell cycle regulatory events [5]. Phosphorylation provides a means of rapidly increasing or decreasing the activity, half-life or redirecting the localization of proteins and is essential for regulating a multitude of biological events [6]. There is now increasing evidence that many of the substrates for regulation by phosphorylation are key enzymes in basic metabolic pathways, including those of nucleotide biosynthesis. Hence one of the remaining challenges is to decipher the networks of signaling pathways that regulate

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these important enzymes and have the potential to modulate the availability of pyrimidines for growing cells. In this review we will discuss the state of knowledge regarding the interactions between growth factor-mediated signal transduction with enzymes of the pyrimidine biosynthetic pathway.

Because of the extensive amount of information on pyrimidine synthesis in mammals, we have chosen to focus this article on regulation of the de novo pathway, culminating in the formation of the major pyrimidine triphosphates UTP and CTP. For excellent reviews of pyrimidine nucleotide synthesis by the alternative salvage pathway or the extracellular effects of uridine nucleotides, the reader is referred to the following review articles [7–10]. In some instances we have diverged to discuss advances in simpler organisms that may provide important examples of how regulation is achieved in mammalian cells. We have chosen to illustrate recently elucidated aspects of regulation and have focused on the key enzymes in this pathway, the fates of the products of this pathway and some of the potential consequences of pyrimidine synthesis on cellular proliferation, differentiation and death.

General overview of the de novo pyrimidine biosynthetic pathway in mammals

The de novo synthesis of pyrimidine nucleotides begins with ATP, glutamine and bicarbonate. The complete conversion of these substrates to uridine requires only three genes and six enzymes, a feat that is accomplished by fusion of multiple enzymatic activities into single polypeptide chains (for a detailed description of this pathway, the reader should consult an excellent review by Jones [11]). The first three steps in the de novo synthesis of pyrimidines are catalyzed by a trifunctional, cytoplasmic enzyme known as CAD, an acronym derived from the names of the three activities in this protein, carbamoyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase.

The unique multienzymic character of CAD was first proposed by Jones on the basis of sequence homologies between the bifunctional Ura 2 and *Neurospora* enzymes [12]. This prediction was confirmed after purification of the enzyme and provided one of the first examples of multiple enzyme activities assembled into a single polypeptide chain [13]. Cloning of the complementary DNA (cDNA) for human CAD confirmed the order of the functional domains of this protein [14], and there are four distinct domains glutaminase (GLNase), carbamoyl phosphate synthetase (CPSase), aspartate transcarbamylase (ATCase) and dihydroorotase (DHOase), with an order GLNase-CPSase-DHOase-ACTase [15]. Transfection of cells lacking CAD activity (G9c) demonstrated that this cDNA was capable of restoring the activities of all three enzymes in a functional manner. Although the basis of the CAD mutation in the G9c cells is unknown, these cells have been extremely useful for facilitating the study of CAD regulation and defining the essential role of the de novo synthesis of pyrimidines [16].

The product of CAD (dihydroorotate) diffuses into the mitochondria, where it is converted to orotate by dihydroorotate dehydrogenase (DHOdehase), the fourth enzyme in the pyrimidine synthetic pathway. Unlike the other enzymes in this pathway that are primarily cytoplasmic, DHOdehase is uniquely situated in the inner mitochondrial membrane, and in parallel with the oxidation of dihydroorotate to orotate, DHOdehase catalyzes the reduction of ubiquinone in the inner mitochondrial membrane [17–19]. Thus the coupling of the DHOdehase catalytic reaction to ubiquinone reduction effectively links the pyrimidine biosynthetic pathway to the mitochondrial respiratory chain, providing the potential for additional mechanisms of regulation [11, 17]. Under some conditions (i.e. CAD overexpression) DHOdehase is likely to become the rate-limiting step in the synthesis of pyrimidine nucleotides. Hence it is not surprising that recent efforts at developing pyrimidine synthesis inhibitors suggest that DHOdehase may be a promising target enzyme for the development of novel drugs (i.e. leflunomide) with antiinflammatory, immunosuppressive or antiproliferative potential [20–23].

The intracellular location of DHOdehase resembles that of NADH dehydrogenase and succinate dehydrogenase of the inner mitochondrial membrane [24, 25]. Recent mutagenesis studies indicate that the extreme N-terminal region of DHOdehase serves as an uncleaved mitochondrial import signal that is unique to the mitochondrially targeted enzymes in mammals. In vitro-synthesized DHOdehase proteins from *Drosophila*, rat and human were efficiently imported into the intermembrane space of isolated yeast mitochondria without proteolytic processing. This required inner membrane potential and was at least partially dependent upon matrix ATP, suggesting that import occurred by previously described mechanisms. Moreover, deletion of specific sequences within the N-terminus of DHOdehase demonstrated that this region contained a bipartate signal that was required for both the import and insertion of this protein into the mitochondrial membrane [19].

The final two steps in this pathway occur in the cytoplasm and are catalyzed by the bifunctional enzyme orotate phosphoribosyltransferase/orotidine-5′-monophosphate decarboxylase (also known as UMP synthetase) which culminates in the synthesis of UMP. While UMP synthetase is required for the completion of the de novo synthetic pathway, this bifunctional enzyme also contributes to the salvage of orotate [11]. UMP is the precursor for all other

pyrimidine nucleotides. The corresponding di- and triphosphorylated forms of UMP (UDP and UTP) are formed by the action of UMP/CMP kinase and nucleoside diphosphate kinase; these phosphorylated bases are required for the subsequent synthesis of the deoxy pyrimidine nucleotides and UDP sugars, respectively (see fig. 1). UTP also provides the entry point for the synthesis of the other major pyrimidine nucleotide, cytidine triphosphate (CTP). CDP (like UDP) is converted to the deoxyribonucleotide derivative by the action of ribonucleotide reductase, which is necessary for synthesis of growing DNA polymers. By contrast, the pyrimidine nucleotides are directly incorporated into chains of RNA and are the precursors for a number of important biosynthetic processes (discussed below).

The phylogenetic roots of the pyrimidine synthetic pathway can be traced back to simple organisms and demonstrate the mosaic nature of the evolution of this pathway [26, 27]. The conservation of multifunctional enzymes in this pathway is observed in higher eukaryotes, trypanosomatids, fungi, dictyostelium and plants to varying degrees of complexity and arrangement that contrasts with that found in prokaryotes (e.g. *Bacillus subtilis*), where single polypeptide chain enzymes are produced, and coordinated regulation is accomplished through clustering of the pyrimidine biosynthetic genes into an operon [28]. Hence, the tethering of multiple enzymatic activities into single polypeptide chains may have evolved to facilitate the efficient conversion of labile intermediates into products in higher eukaryotes.

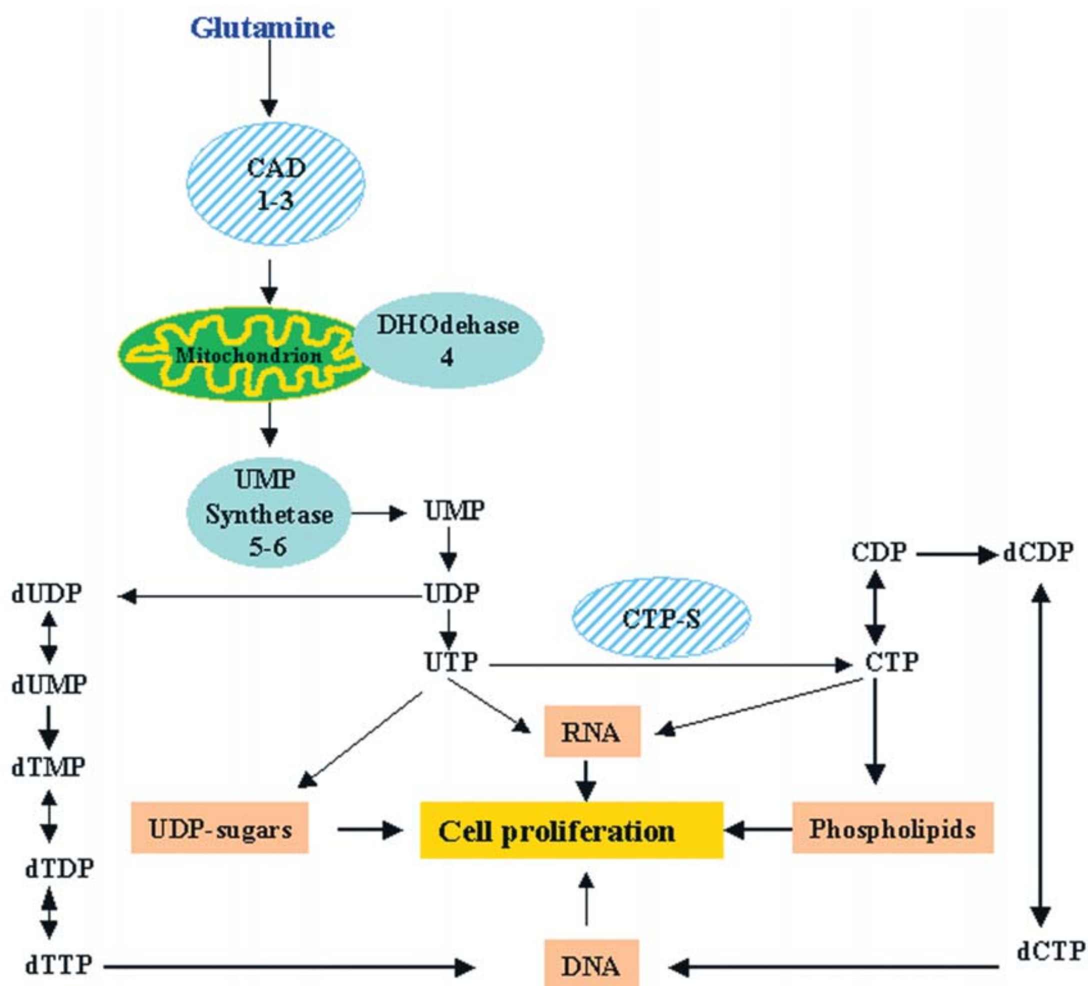

Figure 1. Model of pyrimidine synthesis by de novo pathways. A model is depicted showing the de novo pyrimidine nucleotides synthesis in mammalian cells [11, 134]. The de novo synthesis of pyrimidine nucleotides requires six enzymes. The first three (1–3) are catalyzed by a trifunctional, cytoplasmic enzyme known as CAD, an acronym derived from the names of the three activities in this protein, carbamoyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase. The fourth enzyme (4) is a mitochondrial enzyme, dihydroorotate dehydrogenase (DHOdehase). The last two steps (5–6) are cytoplasmic and are catalyzed by UMP synthetase, the bifunctional enzyme orotate phosphoribosyltransferase/orotidine-5′-monophosphate decarboxylase. CTP synthetase (CTP-S) catalyzes the rate-limiting step which converts UTP to CTP. CAD and CTP-S are highlighted (grid) to emphasize the rate-limiting enzymes in the synthesis of uridine and cytidine nucleotides, respectively.

Allosteric regulation of the pyrimidine synthesis pathway

As commonly observed in biology, the first committed step to a complex pathway is highly regulated and rate limiting. Under normal conditions, the CPSase (CPS II) of CAD catalyzes the rate-limiting step in the de novo pyrimidine synthetic pathway [11, 24]. While the crystal structure of this multifunctional enzyme has not been solved, the corresponding CPSase from *Escherichia coli* has been elucidated, and the reader is directed to a number of elegant reviews on this topic [29–31]. Not unexpectedly, the activity of this enzyme is regulated by a number of different mechanisms, including allosteric and transcriptional regulation, phosphorylation and selective degradation. Moreover, some of these mechanisms may influence each other to coordinately regulate CAD. For example, phosphorylation has been shown to affect the allosteric regulation of CAD by the effectors UTP and PRPP, and the influence of these diverse regulatory mechanisms on CAD is discussed below.

Allosteric modulation of the enzyme activities within the de novo pyrimidine biosynthetic pathway is one of the major mechanisms by which flux through this pathway is controlled in mammalian cells. The CPS II activity of CAD is the site of allosteric activation by ATP or phosphoribosyl pyrophosphate (PRPP) and inhibition by uridine or cytidine nucleotides [11, 24]. PRPP also increases the activity of the 5th and 6th enzymes of the pathway, the bifunctional enzyme orotate phosphoribosyltransferase/orotidine-5′-monophosphate decarboxylase (also known as UMP synthetase). Because PRPP is also an essential precursor for the synthesis of purines, increases in PRPP levels may function to coordinate the parallel synthesis of purines and pyrimidines in mammalian cells.

Conversely, pyrimidine nucleotides inhibit the CPSase activity of CAD in a classical 'feedback' inhibitory manner [11, 24]. Inhibitors of CPS include the phosphorylated derivatives of uridine (UMP, UDP, UTP), cytidine (CTP) and sugar derivatives (UDP glucose). Thus feedback inhibition may provide a mechanism to adjust the synthesis of pyrimidine nucleotides to meet cellular needs and prevent excessive synthesis of nucleotides. This hypothesis is supported by studies showing that mutations in CPS that lose feedback inhibition accumulate large amounts of pyrimidine nucleotides. A missense mutation in the CAD homologue in *Drosophila melanogaster* (Rudimentary), known as suppressor of black [Su (b) or rSu (b)], results in loss of feedback inhibition by UTP and the accumulation of large amounts of this nucleotide [32].

Synthesis of CTP from UTP

In the de novo pathway, the conversion of uridine to cytidine nucleotides is accomplished by a highly regulated enzyme, CTP synthetase [33, 34]. The activity of this enzyme has been shown to be substantially higher in tumor cells [35] and is regulated by phosphorylation in *Saccharomyces cerevisiae* (more on the regulation of this enzyme below). In addition to changes in the synthesis of pyrimidines (UTP), the formation of CTP may also be influenced by changes in cellular purines since ATP is a required substrate and GTP is an allosteric activator of CTP synthetase. Thus the regulation of CTP synthesis may also be responsive to changes in purine nucleotide levels [11, 24].

The synthesis of CTP is essential for a number of biosynthetic processes [34]. One of these is the CTP-dependent synthesis of phosphatidylcholine (PtdCho) and the lipid second messengers derived from this molecule (fig. 1). In addition, CTP lipids are essential for the incorporation of fructose and mannose into GDP sugar precursors. The pool of free CTP is typically the smallest of the triphosphorylated nucleotides, and data suggest that changes in the levels of this nucleotide may have significant influence on the regulation of cell proliferation [7]. Recent studies using either inhibitors of the de novo pyrimidine synthetic pathway (e. g. leflunomide) or uridine starvation of cells lacking a functional CAD protein (G9c) further support the importance of this nucleotide in cell cycle progression [36].

Cellular fates of pyrimidine nucleotides

UDP-sugar synthesis and glycosylation

Uracil nucleotides are essential for the synthesis of nucleotide sugars such as uridine diphosphoglucose (UDP glucose) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) [37]. Nucleotide sugars are synthesized in the cytosol and transported to the endoplasmic reticulum or Golgi compartments, where they are utilized for glycosylation reactions. UDP-glucose originates from the action of UDP-glucose pyrophosphorylase on UTP and glucose 1-phosphate and is the precursor for a variety of UDP-sugar derivatives, including UDP galactose, glucuronic acid, xylose, iduronic acid and so on [37, 38]. Ultimately, these compounds are utilized for the synthesis of disaccharides and complex polysaccharides such as glycogen in mammalian cells. UDP-GlcNAc is synthesized by the hexosamine pathway and is required for the formation of glycosylation of proteins and the attachment of glycosylphosphatidylinositol anchors. Hence the availability of uracil nucleotides could impact these important biosynthetic processes, and recent studies show that reduced UDP-GlcNAc synthesis influences both cell cycle progression and susceptibility to apoptosis [39]. Cytidine nucleotides are also required for the posttranslational modification of proteins, and the synthesis of CMP-neuram-

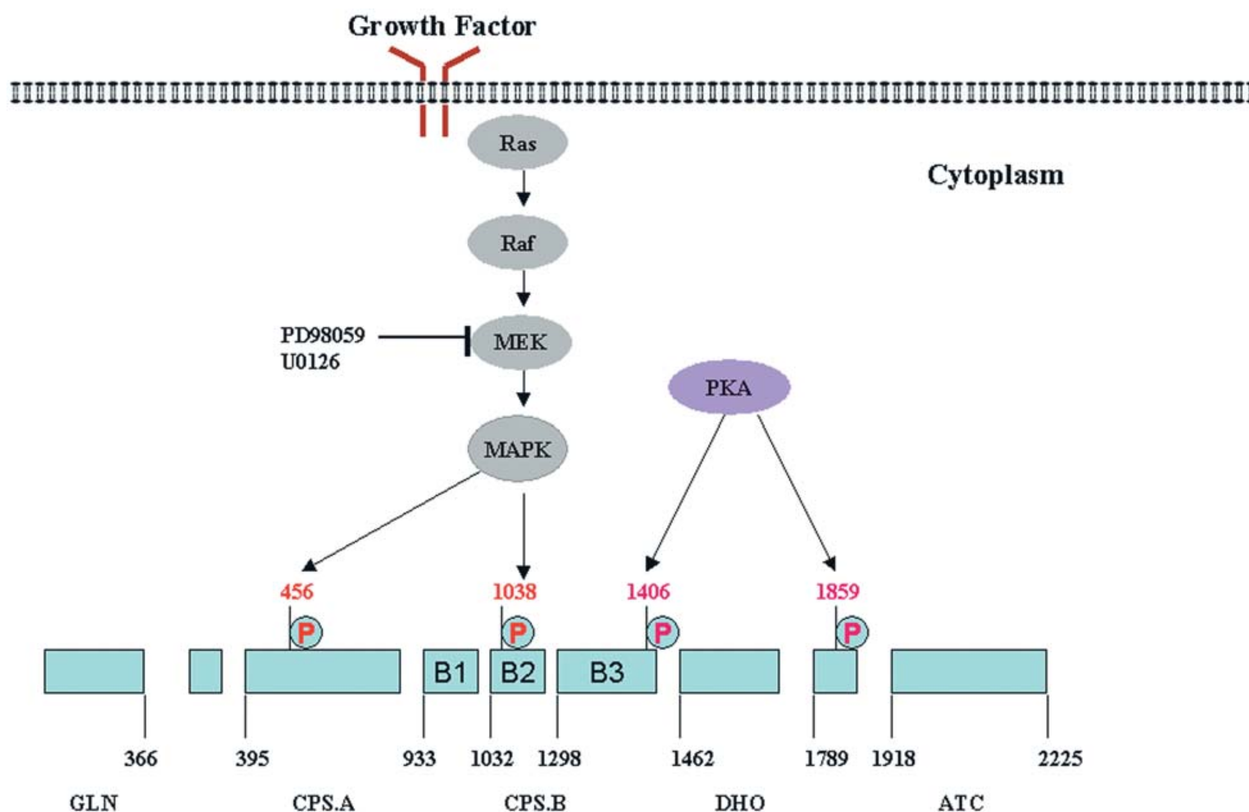

Figure 2. Diagram of potential MAPK and PKA phosphorylation sites in CAD. The growth-factor-activated cascade and proposed MAPK and PKA phosphorylation sites in the mammalian CAD protein. The overall domain structure of the enzyme is shown. GLN represents the glutaminase domain, CPS. A, CPS; B, the tandem carbamoyl phosphate synthetase domains; DHO, the dehydrooratase domain and ATC, the ATCase domain. Amino acid labeling is based on the hamster cDNA [16].

inate (CMP-NANA) is essential for protein sialyation [40, 41].

Although the existence of distinct nucleotide pools has been debated [7], there is evidence that the origin of the nucleotide may play a role in dictating the fate of the nucleotide. The results of one study indicated that pyrimidines incorporated into UDP sugars occurred primarily from the de novo biosynthetic pathway, whereas those derived from the salvage pathway were preferentially directed into RNA [38]. Additional studies have supported the channeling of UTP derived from orotate into UDP-sugar formation in hepatocytes and rat liver [42, 43]. By contrast, the mitogen-induced increase of pyrimidine nucleotides or UDP-glucose was shown to occur by both the de novo and salvage pathways in T lymphocytes [44]. Thus, whether distinct pools of nucleotides have different endpoints remains to be resolved.

RNA and DNA synthesis

Pyrimidine synthesis is required for the formation of both RNA and DNA and will not be discussed in detail here. The synthesis of RNA occurs primarily in the nucleus and has been proposed to originate from distinct pools of nucleotides within this organelle [42, 43]. RNA synthesis precedes that of DNA, suggesting an early requirement for pyrimidine nucleotides in this process. UTP and CTP are directly incorporated into growing RNA chains, whereas the bases for DNA synthesis are derived from the corresponding deoxyribonucleotide partners (dTTP and dCTP). Conversion of the ribonucleotides to deoxyribonucleotides occurs at the level of UDP and CDP and is catalyzed by ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis [11]. dCDP is converted to dCTP in a single step by nucleoside diphosphate kinase, while metabolism of dUDP to dTTP requires a minimum of four additional steps, including that catalyzed by thymidylate synthase. Once formed, dTTP and dCTP provide the substrates for polymerization of DNA (fig. 1).

PtdCho synthesis

PtdCho is the most abundant lipid in eukaryotic cells. In addition to its role in membrane synthesis, it provides the reservoir for numerous lipid derivatives, including lysoPtdCho, phosphatidic acid, diacylglycerol, lysophosphatidic acid, platelet-activating factor and arachidonic acid [34]. These derivatives also have important roles as lipid second messengers and thereby influence multiple cellu-

lar signaling processes. Although there are two established pathways by which PtdCho can be synthesized, in mammals the CDP-choline pathway is the primary synthetic route, whereas yeast (*S. cerevisiae*) rely more on the phosphatidylethanolamine-methylation pathway. The CDP-choline pathway is initiated by CTP synthetase, the enzyme that converts UTP to CTP through an ATP-dependent glutamination reaction. Studies by Vance and colleagues demonstrated that the rate-limiting enzyme in PtdCho synthesis (CTP: phosphocholine cytidyltransferase) is regulated by the concentration of CTP in cells [45, 46]. Thus the biosynthesis of PtdCho and the subsequent phospholipids derived from lipid is absolutely dependent on CTP synthesis in mammals.

Considerable evidence now supports a role for PtdCho synthesis in modulating cell cycle progression and cell proliferation [47]. Conversely, inhibition of PtdCho synthesis is sufficient to induce cell cycle arrest and apoptosis [48]. There is a rapid inactivation of PtdCho synthesis during apoptosis, suggesting that uncoupling of this process is part of the cell death program [49]. Predictably, the synthesis of PtdCho is one of the essential growth-related endpoints of the pyrimidine pathway that influences cell viability and fate.

Synthesis of pyrimidine nucleotides by salvage pathways

In mammalian cells, pyrimidine nucleotides are also synthesized by an alternative salvage pathway, a process that requires the facilitated transport and subsequent phosphorylation of uridine and cytidine to form UMP and CMP, respectively [8]. A variety of membrane transporters are responsible for the transport of nucleosides and include the equilibrative (sodium-independent) and concentrative (sodium-dependent) class of transporters [50]. The equilibrative transporters are further defined by sensitivity or insensitivity to pharmacological agents such as nitrobenzylthioinosine (for excellent reviews on nucleoside transporters, the reader is referred to [51–54]). Once inside the cell, uridine or cytidine are phosphorylated by uridine/cytidine kinase (UCK). There are least two isozymes of this protein UCK1/2 that catalyze this event, as well as the phosphorylation of numerous uridine and cytidine analogues [55]. Studies suggest that UCK is regulated in response to growth factors and is markedly increased in various tumor cell lines [56]. However, to date the majority of these studies suggest that the increase in UCK activity occurs from increased protein expression, and no evidence for the phosphorylation-dependent regulation of this enzyme has been reported.

An alternative route includes salvage of exogenous orotate by the action of UMP synthetase [57]. The extent that de novo or salvage pathways contribute to the expansion of pyrimidine pools may vary considerably between cell types and also depend on the availability of uridine. For instance, in rapidly growing cells such as lymphocytes activated by phorbor esters (PMA), lipopolysaccharide (LPS), and tumor necrosis factor-α (TNF-α) or regenerating liver, the nucleoside transport activity is rapidly upregulated with a concomitant increase in the de novo pathway, suggesting an enhanced requirement for pyrimidine nucleotides [44, 58]. In comparison, in fully differentiated cells such as cardiomyocytes and skeletal muscle tissue, the utilization of the salvage pathway probably exceeds that of the de novo pathway [59]. This reduced reliance on the de novo pathway in more highly differentiated cells may reflect the slower synthetic rate and a reduced requirement for uridine and cytidine nucleotides of these cells [7].

It has long been known that uridine phosphorylase (Upase) regulates the plasma concentration of uridine through phosphorolysis of uridine [44, 60], however, recent studies have shown that Upase can play an unexpected, anabolic role in 5-fluorouracil (5-FU) activation [61, 62] and pyrimidine salvage by catalyzing the synthesis of uridine from uracil and ribose 1-phosphate in rat liver, brain extracts or PC12 cells [10, 63–65]. Subsequent phosphorylation by uridine kinase completes the salvage synthesis by this route.

Regulatory control mechanisms of the pyrimidine biosynthetic enzyme

Regulation of CAD by transcription and translation

Myc-dependent regulation of CAD expression

C-myc (myc) is a sequence-specific family of transcription factors that play a pivotal role in the regulation of cell proliferation. Mutations in myc demonstrate the oncogenic potential of this protein, and myc has been shown to be frequently overexpressed in human tumors [66, 67]. Moreover, the ability of myc to cooperate with Ras to increase transformation of cells is well established [68]. Myc expression peaks in dividing cells and is suppressed as cells exit the cell cycle or terminally differentiate. Overexpression of myc can also trigger apoptosis in some cells [69]; however, the mechanisms by which myc controls growth, tumorigenesis or death is complex and not well understood.

There is now compelling evidence for a connection between myc and CAD expression. Initially, a strong correlation between growth signals that regulate myc and CAD messenger RNA (mRNA) expression was observed. Cloning of human and hamster CAD promoters indicated the presence of canonical myc binding, E-box sequences that were capable of directly binding to myc [70]. Mutation of these sequences or the expression of inhibitory myc mutants blocked growth-factor-stimulated increases in CAD promoter activity, thus de-

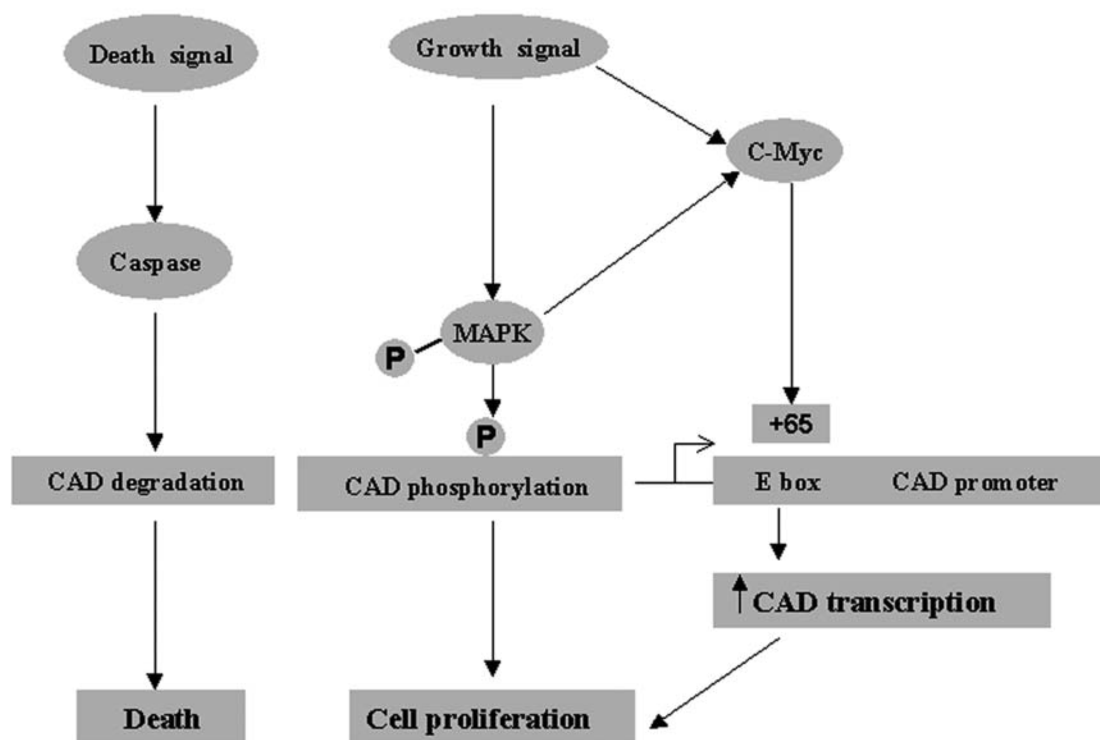

Figure 3. Model of CAD regulation by phosphorylation-targeted degradation or transcriptional control. Potential regulation of CAD by growth-dependent transcriptional or phosphorylation-dependent mechanisms, and control of CAD by caspase-dependent degradation during death

monstrating that CAD expression was dependent on both myc and the intact E-box sequences (fig. 3) [71, 72]. Furthermore, examination of CAD expression in Myc nullizygous cells demonstrated that of all the proposed target genes for myc, only the CAD and GADD45 genes showed loss of growth regulation in rat cells nullizygous for myc [73]. Thus these studies demonstrate that myc-dependent increase in CAD expression is a mechanism by which the activity of this enzyme is increased in tumor cells.

Myc is a well-known target of regulation by phosphorylation and heterodimerization [66]; however, how these processes impact CAD regulation has not been investigated. There is evidence that myc is phosphorylated in response to activation of the mitogen-activated protein kinase (MAPK) cascade [74], and this event could facilitate cooperation between myc and Ras signaling (fig. 3). Although CAD protein is overexpressed in many tumor cell lines [75], additional studies suggest that regulation by phosphorylation may also contribute to control of this enzyme [76].

Conversely, a marked decrease or downregulation of myc has been reported in association with terminal differentiation in lines of human promyelocytic HL60 cells [77], murine erythroleukemic MEL cells [78] and murine F-9 teratocarcinoma cells [79]. The decrease in myc mRNA levels occurred rapidly during the early events of cell commitment and prior to the first sign of terminal differentiation, suggesting that the downregulation of myc may be instrumental in preparing the cells for terminal differentiation. Treatment of the chronic myeloid leukemia (CML) blast crisis K562 cells which express the p210 BCR-ABL protein with cytosine arabinoside (Ara-C) results in downregulation of myc prior to the onset of terminal differentiation [80]. Concomitantly, the levels of myc bound to the CAD promoter were greatly reduced after differentiation [81]. Therefore, decreased synthesis of CAD may occur in parallel with the downregulation of myc during the differentiation of leukemia cells and thereby contribute to the procession of cell differentiation.

Regulation of CAD by phosphorylation; influence on the allosteric properties

Phosphorylation is responsible for the acute regulation of numerous proteins that catalyze important growth-related events [82]. The MAPK cascade is one member of a highly conserved family of kinases capable of transmitting phosphorylation-dependent signals to transcriptional, translational and other enzymatic processes in cells [4]. The MAPKs (also known as Erk1/2) have been reported to phosphorylate multiple substrates and recognize sequences containing a Ser/Thr-Pro motif [83]. Data from our laboratory demonstrated that the multienzyme com-

plex CAD was phosphorylated by MAPK in vitro and in response to growth factors (EGF, PDGF) that activated MAPK in vivo (fig. 2) [76]. The EGF-dependent phosphorylation was prevented by incubation of cells with a chemical inhibitor of the MAPK cascade, PD98059 [84]. Studies performed in collaboration with the Evans laboratory indicated that MAPK-mediated phosphorylation influenced the allosteric regulation of CAD by UTP and PRPP [76]. Specifically, a loss of feedback inhibition of CPSase by UTP and a gain of allosteric activation by PRPP was observed in parallel with phosphorylation by MAPK or treatment with EGF. Incubation of cells with PD98059 reversed the effects of EGF and mutation of a potential MAPK phosphorylation site (Thr 456) abolished the growth-factor (PDGF)-dependent regulation of CAD in G9c cells [76]. However, whether Thr 456 or other phosphorylation sites are responsible for the altered allosteric regulation of CAD remains to be established. In a recent study, Sigoillot and colleagues provide further evidence for phosphorylation of CAD on Thr using a general phospho-threonine reactive antibody [85]. Analysis of the hamster CAD sequence shows that there are at least 13 potential Thr-Pro phosphorylation sites, and the possibility of MAPK-mediated phosphorylation on serine residues remains. Nano-electrospray mass spectrometry studies from our laboratory shows that MAPK also phosphorylates CAD on Ser 1038 in vitro [J. Han, unpublished observations]. Thus phosphorylation of additional sites in CAD may contribute to the changes in allosteric properties through effects on oligomerization or the binding of allosteric effectors to CAD. Importantly, these studies point to growth-factor – mediated phosphorylation as an additional mechanism of control of CAD activity in the de novo pyrimidine biosynthetic pathway (fig. 2).

CAD was also shown to be a substrate for the cyclic AMP (cAMP)-dependent protein kinase (PKA). Carrey and colleagues originally reported that CAD was phosphorylated at two sites by PKA in vitro [86]. These sites were mapped to the C-terminal end of the 160-kDa GLNase/CPSase domain (site 1: Ser 1406) and the linker between the ATCase and DHOase domains (site 2: Ser 1859), respectively [15, 86, 87]. Phosphorylation of Ser 1406 within the allosteric region of hamster CAD eliminated the allosteric inhibition of CPSase by UTP, and phosphorylation at site 1 (Ser 1406) correlated with the resistance of CPSase to UTP feedback control [15, 86]. Conversely, phosphorylation of CAD by PKA was significantly inhibited in the presence of UTP. When UTP was bound to the protein, PKA phosphorylation was reduced, suggesting that the conformation of the enzyme determined the accessibility of these sites to phosphorylation (reviewed in [15]). CAD phosphorylation was also shown to be phosphorylated in cells after treatment with 8-bromo-cAMP, a PKA activator; this event was prevented by pretreatment with H-89, a PKA inhibitor. Substitution of Ser 1406 with alanine resulted in reduced feedback inhibition of CPSase by UTP without affecting the allosteric activation of CAD by PRPP [87]. However, demonstration that Ser 1406 is a major site of phosphorylation in vivo remains to be established (fig. 2). Comparison of CAD from *S. cerevisiae* suggests that the enzyme from this organism (Ura2) is likely to be regulated by different mechanisms. Although both CAD and Ura2 are multifunctional proteins with considerable sequence homology and a similar domain structure, studies suggest that these enzymes have important regulatory differences. The yeast protein Ura2 is bifunctional enzyme containing CPSase and ATCase activities and a domain (pDHO) which is homologous to mammalian DHOase, but is inactive [88]. Furthermore, lack of conservation of potential phosphorylation sites in the Ura2 protein suggests that this enzyme is regulated differently from the mammalian CAD. For instance, the PKA phosphorylation site 1 in hamster CAD is not conserved in Ura2 (sequence alignment shown in [89]). Phosphorylation of this enzyme from protease-B-deficient, Ura2-transformed cells with PKA in vitro identified a phosphorylation at the expected consensus sequence Arg-Arg-Phe-Ser, corresponding to site 2 in mammalian CAD. Phosphorylation of the Ura2 protein at this site changed neither CPSase nor ATCase activities, nor their sensitivity to UTP, suggesting that the yeast enzyme is regulated differently from the mammalian enzyme [90]. Moreover, the proposed MAPK phosphorylation site in CAD is not conserved in the Ura2 enzyme. Although the threonine residue corresponding to Thr 456 is conserved in this enzyme, the subsequent proline residue is absent, thereby resulting in a site that no longer fits the optimal consensus MAPK phosphorylation motif.

Reciprocal allostery

The term 'reciprocal allostery' was first proposed Carrey and colleagues to explain the observation that the substrates of both the CPSase (CPS II) and ATCase of CAD activate each other [91]. Specifically, the affinity of the ATCase for carbamoyl phosphate and aspartate is modulated when substrate is bound to the CPS II domain; this is proposed to occur through reciprocal conformational changes between the folded CPS II and aspartate transcarbamylase (ATCase) domains. The binding of aspartate to the active site of ATCase causes a conformational change at the active site of CPS II, which protects it from inhibition by its product, carbamoyl phosphate. Reciprocally, the substrates for CPS II affect the active site of ATCase by increasing the affinity for its substrates, endogenous carbamoyl phosphate or the transition-state analogue. Reciprocal allostery justifies the close association of these enzyme activities within a single polypeptide chain and ensures that carbamoyl phosphate is efficiently synthesized and is dedicated to the second step of pyrimidine biosynthesis [92].

Intracellular location

Although primarily believed to be cytoplasmic, studies show that a fraction of CAD may also found in a nuclear localization. A study from Angelleti and Engler provided evidence that in response to adenoviral infection, CAD was specifically associated with the nuclear matrix. Under these conditions, CAD was found in part of a complex with the precursor of the terminal protein (pTP), suggesting that it was recruited to the replication domain of the nuclear matrix [93]. Recent research in spermatazoa have confirmed these observations and provided further evidence for nuclear localization of CAD. In addition, these studies demonstrate CAD immunostaining near mitochondria, suggesting the localization of this enzyme may facilitate the transfer of the CAD product, dihydroorotate, to the mitochondrial enzyme DHOdehase [94] (fig.1). Analysis of the yeast homologue of CAD (Ura2) suggests that a bipartite nuclear localization signal may direct trafficking of this protein to the nucleus. Specifically, mutation of residues within this sequence prevented translocation to the nucleus, and comparison of this putative nuclear localization signal suggests a similar sequence is found in CAD within the region phosphorylated by PKA [90, 95]. However, whether the intracellular localization of CAD is influenced by phosphorylation or other factors remains to be determined.

Analysis of CAD regulation by proteolytic degradation

Consistent with alterations in conformational structure, the proteolysis of CAD appears to be influenced by substrates, allosteric regulators and phosphorylation. Early studies demonstrated that the stability of CAD was improved by inclusion of ATP in buffers during purification [11, 96]. N-terminal sequencing of proteolytic fragments has confirmed predictions that the most accessible residues were found in the region linking the aspartate transcarbamoylase and dihydroorotase domains and suggested that this region was hypersensitive to proteinase action in vitro and in vivo [97].

Mammalian CAD protein also was shown to be resistant to proteolysis in the presence of the allosteric inhibitor UTP, suggesting that the conformation of CAD in the presence of UTP was more compact than the unliganded form [96]. Photoaffinity labeling of the *E. coli* CPSase with [^{14}C] UMP specifically prevented trypsin cleavage at a conserved residue, Lys993 [89], and defined the dinucleotide fold region in CPSase as a region responsible for binding the allosteric inhibitor UTP. Phosphorylation of CAD with PKA accelerated the cleavage of native CAD by both elastase and trypsin, and abolished the protective effect of UTP. PKA phosphorylation (site 1) occurs at a residue located close to the C-terminal end of the 160-kDa GLNase/CPSase region, and there are also one or more previously undetected minor phosphorylation site(s) located in the protease-sensitive hinge region between the DHOase and ATCase domain [15]. Thus these results suggest that phosphorylation induces a generalized alteration in the conformation of CAD that exposes the interdomain regions to proteases [96, 98].

Regulation of CAD degradation during apoptosis and cell differentiation

Despite considerable knowledge of the susceptibility of CAD to proteolytic degradation in vitro, few studies have investigated the stability of this enzyme in vivo. By contrast, the highly selective degradation and inactivation of ATCase and other nucleotide biosynthetic enzymes has been demonstrated during sporulation of the prokaryotic organism *B. subtilis* [99]. The normal turnover rate of CAD is estimated to be fairly slow, with a half-life of approximately 24 h [100, 101]. However, the level of CAD expression and activity may be adjusted to meet declining pyrimidine demands in more highly differentiated cells. Research on *Drosophila* has demonstrated that the expression of CAD (Rudimentary) is highly regulated during development. The highest level of activity is found in the early larval stages and declines rapidly thereafter [102].

Recent studies from our laboratory show that CAD is a target for caspase-mediated degradation during cell death [103] (fig. 3). Caspases are a specific class of proteases that are activated during programmed cell death (apoptosis) in eukaryotic cells. Caspases cleave their peptide substrates on the C-terminal side of aspartic acid residues with slight differences in substrate specificity [104, 105]. The proteolysis of caspase substrates is believed to accelerate or coordinate the progression of apoptosis by inactivation of important cellular targets [106]. We found that CAD was rapidly degraded early in the progression of apoptosis and identified two caspase-3 cleavage sites (EAVD/G and VACD/G) located in the catalytic domain and allosteric regulatory domains of CAD, respectively (fig. 2). Cleavage at these sites resulted in a loss of CPSase activity and allosteric activation by PRPP. In parallel with the inactivation and degradation of CAD, cellular pyrimidine levels dropped during the initial stages of apoptosis [103]. Thus these studies suggested that the selective removal of CAD was important for uncoupling this growth-related biosynthetic process during cell death. Identification and mutagenesis of additional caspase cleavage sites may ultimately allow determination of the impact of CAD degradation on the progression of apoptosis.

CTP synthetase

CTP synthetase is the rate-limiting enzyme in the synthesis of cytosine nucleotides [33, 107, 108]. CTP synthetase catalyzes the ATP-dependent transfer of the amide nitrogen from glutamine to the C-4 position of UTP to form CTP [109]. Regulation of CTP synthetase activity controls the balance of the pyrimidine nucleotide pools [110, 111] and influences the pathways by which membrane phospholipids are synthesized [112–114]. Increased CTP synthetase activity is commonly observed in leukemic cells [115–117] and in rapidly growing tumors of liver [35], colon [118] and lung [119]; this observation led to the development of a highly specific inhibitor of this enzyme, cyclopentenyl cytosine (CPEC) [120]. Despite early, serious toxicity problems [121], more recent studies suggest that this compound may still hold promise as a potential antineoplastic [122–125] or antiviral agent [126].

In mammals, two genes encoding human CTP synthetase (type 1 and 2 human CTP synthetase) have been identified and located to chromosomes 1p34 and Xp22, respectively. The predicted protein sequence showed 74% identity with the translation product of the previously identified cDNA coding for type 1 human CTP synthetase. The two isoforms of human CTP synthetase showed a comparable identity (44–55%) to the two isoforms of CTP synthetase from *S. cerevisiae* [33]. At present it is not known which isoform of CTP synthetase is predominant in normal (resting), proliferating and malignant cells. However, since CHO cells deficient in type 1 CTP synthetase required cytidine for growth [127], it seems unlikely the type 2 CTP synthetase is the major enzyme for the synthesis of cytidine nucleotides in these cells [33].

In *S. cerevisiae*, two isoforms of CTP synthetase have been identified and are encoded by the URA7 and URA8 genes [110, 111]. The deduced protein products of the URA7 and URA8 genes contain a conserved glutamine amide transfer domain common to CTP synthetases from other organisms. The URA7-encoded CTP synthetase is more abundant than the URA8-encoded enzyme [113] and is responsible for the majority of the CTP synthesized in vivo [111]. Both of these enzymes are regulated by CTP product inhibition [128, 129], which is believed to regulate the cellular concentration of CTP in growing cells [112, 113, 128]. A glutamate-to-lysine (E161K) mutation in the URA7-encoded CTP synthetase rendered the enzyme defective in regulation by CTP product inhibition, and cells expressing this mutant enzyme exhibited elevated levels of CTP and alterations in phospholipid metabolism [112].

Regulation of CTP synthetase by phosphorylation

Extensive studies of CTP synthetase in yeast have defined phosphorylation as a mechanism by which this activity is controlled in this organism. Specifically, the phosphorylation of the URA7-encoded CTP synthetase by PKA and protein kinase C (PKC) results in the stimulation of activity and causes a decrease in the sensitivity of the enzyme to CTP product inhibition [130, 131]. Similar regulation of this enzyme was observed after phosphorylation by these kinases in vitro [130–132]. In vivo, the URA7-encoded CTP synthetase is phosphorylated at multiple sites, and this event appears to be mediated by the RAS/cAMP pathway [131, 132].

The deduced amino acid sequence of the URA7-encoded CTP synthetase has one potential target site for PKA and eight potential target sites for PKC; however, phosphopeptide mapping experiments demonstrate that the PKA and PKC sites were distinct [130–132]. One of these sites, Ser 424, has been recently identified as the target site for PKA, and phosphorylation of this site has shown to regulate CTP synthetase activity [133]. Mutagenesis of serine to alanine (S424A) demonstrated that this enzyme was not phosphorylated or affected by the activation of PKA in vivo. Specifically, the S424A mutant CTP synthetase had a reduced V_{max} and elevated K_m values for ATP and UTP as well as increased sensitivity to CTP product inhibition when compared with the PKA-phosphorylated wild-type enzyme [133]. Moreover, recent studies suggest that CTP synthetase may be phosphorylated by additional protein kinases or phosphorylated in a hierarchical manner [112, 131, 133]. Preliminary studies have shown that the URA7-encoded CTP synthetase was also phosphorylated by casein kinase II; however, neither the PKC nor the casein kinase II phosphorylation sites in the enzyme have been identified [133]. Whereas the biochemical characterization and regulation of CTP synthetase has been extensively studied in yeast, no such studies have been reported regarding the mammalian enzyme. Moreover, the typical PKA sequence motif in the URA7-encoded CTP synthetase is missing in the mammalian CTP synthetase, suggesting that the important regulatory mechanisms of the mammalian enzyme remain to be identified.

Regulation of CTP synthesis by tetramerization of CTP synthetase

Ligand-induced oligomerization is an important mechanism of regulation of many key metabolic enzymes [134], and recent studies showed that tetramerization was required for CTP synthetase activity [131, 135]. URA7- and URA8-encoded CTP synthetases exhibit positive cooperative kinetics with respect to UTP and ATP binding [128, 129]. This cooperativity has been attributed to the nucleotide-dependent oligomerization of the dimeric form to the tetrameric form [128, 129]. UTP and CTP serve as substrates in the CTP synthesis reaction, and they are responsible for the tetramerization of the enzyme [135]. URA7-encoded CTP synthetase exists as a dimer in the absence of ATP and UTP, whereas in the presence of sat-

urating concentrations of ATP and UTP it is found as a tetramer. CTP, also a potent inhibitor of CTP synthetase activity, does not inhibit the ATP/UTP-dependent tetramerization of the enzyme. Thus, CTP inhibits the activity of CTP synthetase when the enzyme is in the tetrameric form [135].

Phosphorylation of CTP synthetase is believed to regulate the oligomeric form of the enzyme [131, 135]. Phosphorylation of purified CTP synthetase by PKA and PKC facilitates the nucleotide-dependent tetramerization of the enzyme [131, 135]. Dephosphorylation of CTP synthetase with alkaline phosphatase prevented the nucleotide-dependent tetramerization of this enzyme and was correlated with the inactivation of CTP synthetase activity. Phosphorylation of the dephosphorylated enzyme with PKA and PKC resulted in a partial restoration of tetramerization and enzyme activity [135].

Relationship of the pyrimidine biosynthetic pathway to cell proliferation, differentiation and development

Proliferation

Multiple lines of evidence argue for an essential role of pyrimidine nucleotides in influencing the rate of cell proliferation during development. The majority of these studies are based on pharmacological inhibitors or genetic mutations that prevent the normal accumulation of pyrimidine nucleotides in cells. For instance, there is significant evidence for the role for pyrimidine nucleotides and the regulation of CAD in the development of organisms such as *Drosophila* [136]. Mutations in *Drosophila* CAD (rudimentary) or treatment of larvae with PALA (an inhibitor of CAD), resulted in a stubby or shortened wing phenotype [137]. These studies demonstrated that the inability to provide sufficient pyrimidines limited imaginal disc cell proliferation and normal wing development. Interestingly, this phenotype is similar to that found with Ras mutants [138], and further illustrates the demand for pyrimidines during rapid cell growth and development. Studies also show that the levels of CAD activity change substantially during *Drosophila* larval development. The CPS II activity of CAD activity is disproportionally low initially, but increases rapidly during the first instar stage of development. As the larvae develops, total CAD activity declines sharply [102, 139, 140]. However, the mechanisms by which pyrimidine synthesis is regulated during development are not well understood and may reflect changes in Myc expression, phosphorylation, or the degradation of CAD or other enzymes in this pathway.

The importance of pyrimidine nucleotide availability in regulating cell proliferation in mammalian cells is well recognized. Aside from providing for the basic requirements in resting cells, pyrimidine nucleotide pools fluctuate in response to cellular demands during cell proliferation. Compared with the concentration of intracellular ATP in normal cells, the amounts of intracellular UTP and CTP are relatively small [7]. In human T lymphocytes, a remarkable expansion of cellular pyrimidine pools has been observed following PHA stimulation. Noticeably, the pyrimidine nucleotide pools increased up to eight-fold (UTP, UDP-Glc and CTP) pools, whereas the purines (ATP and GTP) increased only approximately two-fold [44]. This disproportionate expansion of pyrimidine pools in T lymphocytes suggests a unique requirement for UTP and CTP in growth-related processes, such as lipid and protein glycosylation, and membrane biosynthesis as discussed above. The increase in pyrimidine pools in response to mitogen treatment was shown to occur primarily through increased flux through the de novo pathway, although some increase in the salvage pathways was also observed in these experiments [44]. Alterations in pyrimidine synthesis were also found in human leukemic cells through increases in both de novo and salvage pathway synthesis [141].

Further evidence for the requirement of pyrimidines during growth comes from the comparison between normal and transformed cells. In normal cells, the balance between (deoxy)ribonucleotide pools is tightly regulated [142], and the four (deoxy)nucleotide triphosphates must be synthesized in appropriate amounts to maintain normal function for cell viability and proliferation [7]. The levels of pyrimidine nucleotides are expected to change during the cell cycle. In concanavalin A (Con A)-stimulated thymocytes, the maximal incorporation of radiolabeled precursors into nucleotides occurred during the S phase of the cell cycle [143]. In hepatocyte-derived cell cultures, the activities of both de novo and salvage pyrimidine synthetic enzymes increased markedly during log phase growth [144].

Tumor cells have concentrations of 6–11-fold over normal cells for the 4dNTPs (dATP, dGTP, dCTP, dTTP), and for the 4NTPs (ATP, GTP, UTP, CTP), tumor cells have concentrations approximately 1.2–5-fold over the normal cells [7]. Although extensive data show increased synthesis of pyrimidines during cell transformation, there is no evidence that this event directly causes transformation. Rather, multiple studies suggest that this event is permissive for increased cell growth and that inadequate synthesis restricts normal cell proliferation. Not surprisingly, many of the key enzymes of nucleotide metabolism, including CAD [145], CTP synthetase, thymidylate synthase (TS), dihydrofolate reductase, IMP dehydrogenase (IMPDH) and ribonucleotide reductase (reviewed in [2]), are markedly increased in transformed and malignant tumor cells. Combined with additional changes in feedback regulation, the increase in these enzyme activities is predicted to contribute to the overall increase in pyrimidines found in transformed cells.

In addition, the participation of the pyrimidine salvage pathway must also be considered. As cells become more highly differentiated, reliance on the de novo pathway may decline, and utilization of salvage pathway increases. This hypothesis is supported by data demonstrating that in highly differentiated tissues, there is a corresponding decline in the activities of the enzymes of the de novo pathway, whereas the ability of the salvage pathway to synthesize pyrimidines from plasma uridine and cytidine remains [146]. However, the extent to which cells utilize the pyrimidine salvage pathway varies considerably between cell types and may also reflect changes in the activity of uridine kinase, uridine phosphorylase or the nucleoside transporters [10, 54, 57, 147]. In this regard, tissues such as liver, muscle and kidney would be expected to maintain a high capacity for pyrimidine synthesis to provide UTP for glycogen synthesis in these cells.

Differentiation and death

Pyrimidine nucleotides may also be important for the progression of cellular differentiation or death. Inhibition of pyrimidine synthesis with PALA, Brequinar sodium, Leflunomide (LEF) or CTP synthetase inhibitors results in cell cycle arrest, apoptosis and in some instances differentiation [148]. Recent studies from our laboratory demonstrated that LEF treatment of human K562 leukemia cells initiated a specific depletion of pyrimidines, S-phase arrest and erythroid differentiation of these cells [36]. The LEF-induced differentiation of these cells was prevented by coincubation with uridine or cytidine, which restored the pyrimidine pools. Interestingly, restoration of the CTP pool alone (by cytidine salvage) prevented the effects of LEF or uridine starvation of G9cells [36]. Similarly, treatment of K562 cells with the CTP synthetase inhibitor cyclopentyl cytosine (CPEC) also resulted in a potent and selective depletion of CTP, S-phase arrest and erythroid differentiation of these cells. Thus these studies indicate that CTP may be a rate-limiting nucleotide in cell cycle progression and suggest that inadequate levels trigger cell cycle arrest and differentiation.

Concluding remarks

Pyrimidine nucleotides provide essential precursors to multiple metabolic pathways in cells. Recent studies now provide increasing evidence that the rate-limiting enzymes in this pathway are targets for regulation by phosphorylation, degradation and other mechanisms to modulate the availability of pyrimidines to changes in the demands for these nucleotides and their products, during growth differentiation or death. The impact of multiple cellular signaling processes on the regulation of the de novo and salvage pyrimidine synthesis pathways holds promise for an exciting future ahead.

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