Nuclear Localization and Mitogen-activated Protein Kinase Phosphorylation of the Multifunctional Protein CAD*

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Nuclear Localization and Mitogen-activated Protein Kinase

The first three steps of mammalian de novo pyrimidine biosynthesis are catalyzed by CAD1 (1–3), a 1.4-MDa multifunctional protein that carries the glutamine (gln)-dependent carbamoyl phosphate synthetase (CPSase), aspartate transcarbamoylase (ATCase), and dihydroorotase (DHOase) activities (Reactions 1–3).

\[
\begin{align*}
\text{HCO}_3^- & \quad \text{CPSase} \\
2 \text{ATP} & \quad \text{gln} \\
\text{carbamoyl-P} & \quad \text{aspartate} \\
\text{DHOase} & \quad \text{dihydroorotate}
\end{align*}
\]

HCO\textsubscript{3}\textsuperscript{-} \text{CPSase} 2 \text{ATP} gln \text{carbamoyl-P} \text{aspartate} DHOase dihydroorotate

Reactions 1–3

The flux through the pathway is regulated by the CAD CPSase activity, which catalyzes the first and rate-limiting step of the pathway (4, 5). CPSase is subject to feedback inhibition by the end product, UTP, and allosteric activation by PRPP, a substrate of both UMP synthase and glutamine PRPP amidotransferase, the enzyme catalyzing the first step in the purine biosynthetic pathway (6–8). Moreover, the response to allosteric ligands is modulated by phosphorylation via two central signaling cascades (9–11) and by autophosphorylation (12).

Stimulation of resting cells by growth factors, such as EGF and platelet-derived growth factor, activates the MAP kinase (Erk1/2) cascade and triggers the transition to the proliferating stage (13). An important MAP kinase target is CAD. Phosphorylation of CAD Thr-456 by MAP kinase, just prior to entry into the S phase of the cell cycle, increases PRPP activation and converts UTP from an inhibitor to an activator (14). The activity of CPSase is thus stimulated, and the flux through the pathway increases, providing precursors for the synthesis of DNA, RNA, and the glycoproteins needed for membrane biosynthesis.

The activation of the pathway required for cell proliferation is a consequence of the phosphorylation of CAD Thr-456 by mitogen-activated protein (MAP) kinase. Although most of the CAD in the cell was cytosolic, cell fractionation and fluorescence microscopy showed that Thr(P)-456 CAD was primarily localized within the nucleus in association with insoluble nuclear structures, including the nuclear matrix. CAD in resting cells was cytosolic and unphosphorylated. Upon epidermal growth factor stimulation, CAD moved to the nucleus, and Thr-456 was found to be phosphorylated. Mutation of the CAD Thr-456 and inhibitor studies showed that nuclear import is not mediated by MAP kinase phosphorylation. Two fluorescent CAD constructs, NLS-CAD and NES-CAD, were prepared that incorporated strong nuclear import and export signals, respectively. NLS-CAD was exclusively nuclear and extensively phosphorylated. In contrast, NES-CAD was confined to the cytoplasm, and Thr-456 remained unphosphorylated. Although alternative explanations can be envisioned, it is likely that phosphorylation occurs within the nucleus where much of the activated MAP kinase is localized. Trapping CAD in the nucleus had a minimal effect on pyrimidine metabolism. In contrast, when CAD was excluded from the nucleus, the rate of pyrimidine biosynthesis, the nucleotide pools, and the growth rate were reduced by 21, 36, and 60%, respectively. Thus, the nuclear import of CAD appears to promote optimal cell growth. UMP synthase, the bifunctional protein that catalyzes the last two steps in the pathway, was also found in both the cytoplasm and nucleus.
synthesis. As the cells emerge from S phase, Thr-456 is dephosphorylated, and Ser-1406 is phosphorylated by protein kinase A (14). As a consequence, the response to PRPP reverts to the basal level, and the pathway is down-regulated. Thus, the growth-dependent up- and down-regulation of the pathway is governed by the sequential phosphorylation of CAD by the MAP kinase and cAMP-protein kinase A cascades. In accord with this interpretation, the pyrimidine pathway is persistently activated in MCF7 breast cancer cells (15) as a consequence of elevated MAP kinase activity that results in the continuous phosphorylation of CAD Thr-456, whereas CAD Ser-1406 remains unphosphorylated.

Immunofluorescence microscopy using CAD-specific antibodies (16) showed that CAD in BHK cells was primarily localized in the cytoplasmic compartment where it had a granular appearance, suggestive of association with subcellular structures. In human spermatozoa (17), some of the cytoplasmic CAD appeared to be associated with the cytoskeleton and to colocalize to some extent with the mitochondria.

Moreover, small but significant amounts of the protein have been observed to be present in the nucleus of BHK cells (16, 17) and spermatozoa. Furthermore, histochemical assays of ATCase, one of the components of the bifunctional CAD holoenzyme in yeast, showed that this protein is also partially nuclear. Following adenovirus infection of HeLa cells, the viral preterminal protein needed for replication of its genome was found to be anchored in a complex with CAD to the nuclear matrix near the sites of active replication (18). All of these experiments were carried out with fixed cells, but recently a construct encoding CAD fused to a green fluorescent protein variant was transfected into BHK cells (19). CAD was initially localized in the cytoplasm but was translocated to the nucleus during the G2 phase of the cell cycle of synchronized transfectants.

Although the presence of the pyrimidine biosynthetic enzymes in the nucleus during G2 phase to replenish the small, rapidly turning-over deoxyribonucleotide pools is an attractive idea, the other pathway enzymes, DHOdhase and UMP synthase, are thought to be localized outside the nucleus, an observation that undermines the logic of this hypothesis. Thus, an understanding of the physiological significance of nucleocytoplasmic translocation of CAD has been elusive. The studies described in this report showed that CADThr(6)-456 binds to nuclear substructures, that MAP kinase-mediated phosphorylation is not required for nuclear import, that a fraction of UMP synthase is nuclear, and that the initial steps of the pyrimidine pathway can occur within the nucleus.

MATERIALS AND METHODS

Cell Culture—BHK21 cells, a baby hamster kidney cell line, and BHK165-23 (19), a BHK21-derivative cell line that overproduces CAD 100-fold, were grown in DMEM (Invitrogen) supplemented with 10% dialyzed fetal bovine serum and 2 μg/ml gentamicin (Invitrogen). UdA cells, a uridine auxotroph lacking CAD (20), were grown in DMEM/F-12, 8% bovine serum, 30 μM uridine, 2 μg/ml gentamicin. The MCF breast cell lines were a generous gift of Drs. Samuel Brooks, Stephen Santner, and Robert Pauley (Karmanos Cancer Institute, Detroit, MI). MCF10A cells derived from normal breast tissue and MCF7, a cells, a uridine auxotroph lacking CAD (20), were grown in DMEM/H9262 g/mL gentamicin. Human trabecular meshwork cells were isolated from the eyes of 25-35-year-old individuals. Frozen trabecular meshwork cells were a generous gift of Dr. John Alexander, New York Eye and Ear Infirmary, New York City. MCF10A cells derived from normal breast tissue and MCF7, a breast cell line, were grown in DMEM/H9262 g/mL gentamicin. Twenty T75 flasks corresponding to 1–2 × 108 cells were used for the preparation of the total cell extract. The isolated nuclei were washed as described previously (18).

Preparation and Fractionation of Cell Extracts and Organelles—All steps were carried out at 4°C unless otherwise indicated. Cells were washed twice with PBS (Invitrogen) supplemented with 0.2 mM PMSP (Sigma) and removed from the growing surface by scraping. The cells from each flask were resuspended in 4 ml of PBS with 0.2 mM phenylmethylsulfonl fluoride (PMSF). After centrifugation at 5000 × g for 5 min, the packed cells were resuspended at a concentration of 3 × 106 cells/0.2 mL of a lysis buffer consisting of 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10%glycerol, 0.2 mM PMSP supplemented with a 1 × mixture of phosphatase and protease inhibitors (Sigma). The cell lysate was centrifuged 30 min at 23,000 × g. The supernatant was designated the soluble fraction, and the pellet was resuspended in one-half volume of lysis buffer (PMSF). After centrifugation at 5000 × g for 20 min, and then centrifuged for 30 min at 23,000 × g. The supernatant from this second centrifugation step did not contain significant amounts of CAD or phosphorylated CAD.

Nuclei were isolated following a protocol published previously (21). Ten T75 flasks corresponding to 1–2 × 108 cells were scraped and rinsed with PBS supplemented with 0.2 mM PMSF and removed by scraping. The cells were centrifuged at 7,000 × g for 5 min. The cell pellet was resuspended in 5 volumes of 10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and then centrifuged at 2,000 × g for 5 min. The cell pellet was resuspended in 3 volumes of the same buffer, incubated on ice for 15 min, and then lysed in a mixture A Dounce using a loose pestle (10 strokes) was monitored by trypan blue staining. The nuclei were isolated by centrifuging the cell lysate at 4000 × g for 10 min. The presence of the nuclear markers lamin B and C23/nucleolin was verified by using specific antibodies (Santa Cruz Biotechnology). The isolated nuclei were shown to be free of contaminating mitochondria by assaying the DHOdhase activity (22). The dehydrogenase was found only in the insoluble cytoplasmic fraction containing the mitochondria.

The supernatant obtained after pelleting the nuclei was centrifuged at 16,000 × g for 30 min, and the supernatant was designated the soluble cytoplasmic fraction. The pellet was extracted with lysis buffer, and the insoluble cytoplasmic fraction was prepared as described above. The isolated nuclei were washed once in 10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and resuspended in lysis buffer and the soluble and nuclear fractions were prepared as described for the corresponding fractions of the total cell extract. The nuclear matrix was isolated as described previously (18).

For the isolation of the mitochondria (23), 1 × 108 cells were washed as described above and removed from the growing surface with trypsin.

The cells were collected by centrifugation at 4,000 × g for 10 min, washed with 134 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 2.5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 1 × protease inhibitor mixture, and then resuspended in 10 volumes of ice-cold swelling buffer consisting of 10 mM NaCl, 1.5 mM CaCl2, 10 mM Tris-HCl, pH 7.5, and incubated on ice for 15 min. The cell suspension was lysed by 10 strokes in a Wheaton A Dounce homogenizer with an A pestle. Lysis was monitored by trypan blue staining. A 5 × sucrose solution was immediately added to the lysate to give a final concentration of 330 mM sucrose, 6 mM EDTA, and 8.6 mM Tris-HCl, pH 7.5. The lysate was centrifuged twice at 2600 × g for 10 min, and the supernatant fractions containing the mitochondria were pooled and centrifuged at 20,000 × g for 10 min. The mitochondrial pellet was washed once in a 1 × sucrose buffer consisting of 330 mM sucrose, 6 mM EDTA, and 8.6 mM Tris-HCl, pH 7.5. The mitochondrial pellet was washed once in a 1 × sucrose buffer consisting of 330 mM sucrose, 6 mM EDTA, and 8.6 mM Tris-HCl, pH 7.5, and resuspended in Lysis buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 0.2 mM PMSP supplemented with a 1 × mixture of phosphatase and protease inhibitors). As expected, the isolated mitochondria had DHOdhase activity, but no lamin or nucleolin was detected, indicating that the preparation was not contaminated by nuclei.

Construction of Recombinant Plasmids and Mutants—The plasmid pECFP-CAD encoding the entire CAD polypeptide with an enhanced cyan fluorescent protein appended to the amino end (14) served as the parent construct. Two new recombinants were engineered in an attempt to localize CAD in the nuclear compartment or confine it to the cytosol. The construct pECFP-NLS-CAD included three tandem repeats of the nuclear localization signal sequence from the large T antigen of SV40 (24, 25). Oligonucleotides encoding complementary strands of the NLS targeting sequence were synthesized (Invitrogen) as follows: NLS Dimer(Cy5), 5′-ccggattccctacctggagagtctcgggagagagagagacgatagatccggacctatcctggagtctcgggagagagagagacgatagatccggacctatcctggagtctcggagagagagagagacgatagatccggacctatcctggagtct-3′. The com-
pooled oligonucleotides were annealed in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA by heating at 95 °C for 15 min and then allowed to gradually cool to room temperature. The 5' ends of the double-stranded fragments were phosphorylated using T4 polynucleotide kinase (Invitrogen) and 1 mM ATP and then purified with MinElute PCR purification kit (Qiagen). For both constructs, the double-stranded coding sequences were inserted into the BspEI site that lies between the sequences encoding ECFP and CAD of pECFP-CAD. The pECFP-CAD vector was cleaved with BspEI; the singly cut plasmid was isolated and dephosphorylated with thermosensitive alkaline phosphatase (Invitrogen) and repurified using the PCR purification kit. Ligation reactions were carried out using 20 fmol of dephosphorylated linear pECFP-CAD and 200 fmol 5' ends of either NLS or NES double-stranded fragments. The ligation mixture was transformed into competent subcloning efficiency DH5α Escherichia coli cells, and the fidelity of the constructs was verified by restriction digestion and sequencing. Human and DHOdhase and UMP synthase coding sequences were amplified by PCR using as templates the ATCC clones ATCC79564 (pPYRD) and MGC-4244, respectively, and Pfu Turbo DNA polymerase (Stratagene). The PCR products were inserted into the entry vector, pENTR/D/D-TOPO (Gateway System, Invitrogen), following the manufacturer's protocol. Fidelity was verified by nucleotide sequencing. The DHOdhase entry recombinant was subcloned into pcDNA-DEST47 for stable expression as a fusion protein with GST (gift of S. Simmonds, Faculté de Pharmacie, Université Paris-Sud, Orsay, France). Cells incubated with only the secondary antibodies served as a control to assess background fluorescence levels. The images were quantitated using Adobe Photoshop, NIH Image and Meta-morph software.

**Enzyme Assays, Nucleotide Pools, and Pyrimidine Biosynthesis—** Assays of the CAD CPSase, ATCase, and DHOase activities were determined essentially as described previously (14). The DHOdhase activity was assayed by a modification of the methods devised by Huisman et al. (29) and Simmonds and co-workers (30) following a protocol described previously (14). In this assay, the flux through the pathway is measured by the incorporation of [3H]sodium bicarbonate into the CPSase substrate, into UTP and CTP. The radiolabeled and total nucleotides were quantitated by HPLC (14).

**RESULTS**

**Intracellular Distribution of CAD—** ImmunobLOTS probed with CAD antibodies (Fig. 1A) showed that following centrifugation most of the CAD in BHK165-23 cell extracts was soluble, but a significant fraction could be extracted from the insoluble material in the pellet. In contrast, immunobLOTS probed with CAD Thr(P)/456-specific antibodies showed that whereas the phosphorylated protein was present in relatively small amounts in the soluble fraction, most of the phosphorylated protein was found in the insoluble fraction. The BHK165-23 cell line overproduced CAD as a consequence of extensive duplication of the gene; however, the observed partitioning of the protein was not peculiar to this cell line. The same distribution of CAD and phosphorylated CAD (Fig. 1B) was also observed in the normal breast cell line MCF10A, the tumorigenic breast cell line MCF7, human mesothelioma cells CRL2081, human embryonic kidney cells 293, and in baby hamster kidney cells BHK21. Thus, although the bulk of the CAD in the cell extract was soluble, most of the CAD phosphorylated by MAP kinase was found in a particulate fraction that contained crude nuclei, mitochondria, and cytokeratin as determined by immunoblotting with antibodies directed against lamin B, C23 nucleolin, and vimentin and by assaying mitochondrial DHOdhase (see below).

**Fractionation of Cell Extracts—** Previous immunofluorescence microscopy studies (14, 16–18) showed that a fraction of CAD was localized in the nucleus. Nuclei and cytosolic fractions were isolated from BHK21 cells, a cell line containing a normal complement of CAD, and purified following standard protocols (see “Materials and Methods”). Analysis of marker proteins indicated that these fractions were not cross-contaminated (Table I).
The amount of CAD in each fraction was determined by assaying its ATCase and DHOase activities (Table II). These catalytic activities are unregulated and should thus reflect the intracellular concentration of the protein. The amount of total CAD in the cytoplasmic fraction (77%) is significantly higher than that in the insoluble cytoplasmic fraction (23%). The enzymatic assays also demonstrated that an appreciably larger percentage of the CAD is found in the cytosol, the ratio of the phosphorylated to unphosphorylated protein is appreciably lower than CAD bound to the nuclear substructures that are largely phosphorylated.

TABLE I

| Fraction         | Lamin % | Nucleolin | Vimentin | DHOdhase |
|------------------|---------|-----------|----------|----------|
| Cytoplasmic soluble | 0       | 0         | 3.1      | 0.3      |
| Cytoplasmic particulate | 0       | 0         | 96.9     | 92.6     |
| Total cytoplasmic | 0       | 100.0     | 0.0      | 92.9     |
| Nuclear soluble   | 7.8     | 19.3      | 0.2      | 7.1      |
| Nuclear particulate | 92.2   | 80.7      | 0.6      | 7.1      |
| Total nuclear     | 100.0   | 100.0     | 0.0      | 7.1      |

FIG. 1. Partitioning of CAD between soluble and insoluble cell fractions. A, cell extracts of BHK165-23 cells, which overproduce CAD, were prepared and fractionated into soluble (sol) and insoluble (insol) fractions as described under "Materials and Methods." The relative protein concentrations are shown. B, the same experiment was conducted on cell extracts of normal human breast cells (MCF10A), tumorigenic breast cancer cells (MCF7), human mesothelioma cells (CRL2981), human embryonic kidney cells (HEK293), and baby hamster kidney cells (BHK21).

The specific enzymatic activity of CPSase was approximately 3.45 mmol/min/mg (36).

That the extent of phosphorylation of the nuclear insoluble fraction was 3.7-fold greater than that in the soluble cytoplasmic fraction. Thus, by taking into consideration the amount of CAD present in each fraction, the extent of phosphorylation of CAD associated with nuclear substructures was 44-fold greater than cytoplasmic CAD. Phosphorylation of the insoluble cytoplasmic and soluble nuclear subfractions could not be detected. Thus, although there is a significant amount of phosphorylated CAD in the cytosol, the ratio of the phosphorylated to unphosphorylated protein is appreciably lower than CAD bound to nuclear substructures that are largely phosphorylated.

TABLE II

| Cell Fraction         | CPSase activity | CPSase regulation (% of control) |
|-----------------------|-----------------|----------------------------------|
|                       | μmol/g/mg protein | UTP | PRPP |
| Cytoplasmic soluble   | 0.665           | 21.4 | 84.2 |
| Cytoplasmic particulate | 0.603      | 39.2 | 68.8 |
| Nuclear soluble        | 0.405           | 87.7 | 125  |
| Nuclear particulate    | 0.301           | 264  | 374  |

TABLE III

| Cell Fraction | CPSase activity | CPSase regulation (% of control) |
|---------------|-----------------|----------------------------------|
|               | μmol/g/mg protein | UTP | PRPP |
| Cytoplasmic soluble | 5.96             | 81.4 | 85.5 |
| Cytoplasmic particulate | 4.63            | 69.2 | 100  |
| Nuclear soluble   | 5.53            | 87.7 | 125  |
| Nuclear particulate | 4.53            | 264  | 271  |

Activity is expressed per mg of CAD in the fraction as determined by assaying the unregulated ATCase and DHOase activities of CAD.

The distribution of total CAD in the nuclear and cytoplasmic fractions determined by enzyme assays.

![Diagram A](image1)

![Diagram B](image2)

The specific enzymatic activity of CPSase was approximately 3.45 mmol/min/mg (36).
CAD. This series of experiments demonstrated that although the bulk of CAD in the cell is soluble, CAD associated with subcellular structures within the nucleus, including the nuclear matrix, is extensively phosphorylated by MAP kinase.

Confocal Fluorescence Microscopy—The intracellular distribution of CAD was also investigated by confocal immunofluorescence microscopy (Fig. 3). BHK21 cells were fixed and stained with antibodies directed against CAD (Fig. 3A) and were also stained with Hoechst to establish the nuclear boundaries (not shown) and with Mitotracker Red to localize the mitochondria. Analysis of the micrographs (Table IV) showed that the ratio of total CAD in the nucleus to that in the cytoplasm was 0.2, as determined from the integrated intensities. These results are in reasonable agreement with the nuclear-cytoplasmic partitioning of total CAD (0.23:0.77) determined by enzyme assays (Table II). Cytosolic CAD was distributed throughout the cytoplasmic compartment exhibiting the punctate pattern observed previously (16) and appeared to colocalize to a small extent with the mitochondria as reported recently (17). CAD was more uniformly distributed throughout the nuclear compartment.

The same experiment, repeated using (Fig. 3B) Thr(P)-456 CAD antibodies, showed that the partitioning of phosphorylated CAD was dramatically different. The phosphorylated protein in both the nucleus and cytoplasm has a more granular appearance, and in agreement with the biochemical studies described above, a significantly higher density of CAD Thr(P)-456 (P–Thr456) in each subfraction was determined by immunoblotting. B, nuclei, mitochondrial matrix proteins (mito matrix), mitochondrial membranes (mito membr), and the cytosolic fraction (cytosol) were isolated as described under “Materials and Methods” and immunoblotted with CAD Thr(P)-456-specific antibodies and antibodies directed against activated MAP kinase. C, CAD Thr(P)-456 in the nuclear matrix, isolated as described under “Materials and Methods,” was analyzed by immunoblotting. The matrix was suspended in 15 mM Tris-HCl, pH 7.0, 30 mM NaCl, 3 mM MgCl2, and 1 mM DTT and incubated for 1 h at 25 °C. Upon centrifugation at 15,000 × g for 5 min, the phosphorylated CAD remained associated with the pellet containing the insoluble matrix proteins (pellet), and none was released into the supernatant. The same experiment was repeated with 3 mM ATP present in the incubation mixture.

EGF Stimulation Induces Translocation of CAD into the Nucleus—BHK21 cells were deprived of serum for 18 h to arrest their growth. Immunofluorescence microscopy (Fig. 4) showed that CAD was confined almost exclusively to the cytosolic compartment and that Thr(P)-456 CAD was virtually undetectable in both the cytosol and the nucleus. However, stimulation of the resting cells with EGF for 10 min induced the translocation of a significant fraction of CAD into the nucleus. The level of Thr(P)-456 CAD sharply increased, and the phosphoprotein was for the most part nuclear (Fig. 4, A and B).
The partitioning of CAD and Thr(P)-456 CAD between the nuclear and cytosolic compartments determined by fluorescence microscopy

| Protein              | Microscopic method            | Ratio of integrated intensity, a |
|----------------------|-------------------------------|----------------------------------|
| CAD                  | Fixed cells, immunofluorescence| 0.20                             |
| Thr(P)-456 CAD       | Fixed cells, immunofluorescence| 1.91                             |
| CAD Thr-456 → Ala    | Live cells, ECFP-CAD fluorescence| 0.20                              |
| CAD Thr-456 → Glu    | Live cells, ECFP-CAD fluorescence| 0.22                              |

* Micrographs were scanned and analyzed using the Metamorph software. The integrated intensity was calculated by dividing the intensity by the area scanned.

**Nuclear Localization of CAD**

When the resting cells were exposed to the specific MAP kinase kinase (MEK) inhibitor PD98059 prior to EGF stimulation, the amount of Thr(P)-456 CAD was markedly reduced (Fig. 4B), consistent with previous studies (14, 15) that showed that blocking the activation of MAP kinase reduces the phosphorylation of CAD Thr-456. More importantly, PD98059 does not block the EGF-stimulated migration of CAD to the nucleus (Fig. 4A), suggesting that the phosphorylation of Thr-456 is not responsible for the nucleocytoplasmic translocation of the protein.

**CAD Thr-456 Mutants**—To investigate further whether prior phosphorylation of the MAP kinase site is a prerequisite for nuclear translocation, mutants of pECFP-CAD were made in which Thr-456 was replaced with alanine or with glutamate to mimic constitutive phosphorylation. Previous studies indicated that the Thr-456 → Glu mutant altered the allosteric transitions in much the same way as Thr phosphorylation by MAP kinase. Fluorescence microscopy (Table IV) showed that the intracellular localization of both mutants was indistinguishable (nucleus/cytoplasm = 0.19–0.22) from the wild type parent molecule, confirming that MAP kinase phosphorylation is not responsible for the translocation of CAD into the nucleus.

**Trapping CAD in the Nuclear or Cytosolic Compartments**—To investigate the potential functional significance of the nucleocytoplasmic dynamics, constructs were prepared that trapped CAD in the nuclear or cytoplasmic compartments. We had previously constructed (14) a recombinant pECFP-CAD that had the sequence encoding the cyan variant of the GFP protein fused in-frame to the amino end of the CAD coding sequence. The fusion protein, expressed following transfection, was intensely fluorescent, thus allowing the movement of CAD to be monitored in live cells. Two new constructs were engineered. The pECFP-NLS-CAD construct had three copies of the sequence encoding the NLS from SV40 T-antigen inserted between the ECFP and CAD sequences. Similarly, the pECFP-NES-CAD construct has the highly efficient MEK nuclear export sequence. All three constructs were expressed at comparable levels (Fig. 5A) following transfection into Urd A cells. The proteins had the expected molecular mass and cross-reacted strongly with antibodies directed against both CAD and the ECFP protein (not shown). Although the CAD construct was localized primarily in the cytoplasm, the NLS-CAD (Fig. 5, B and C) and NES-CAD (Fig. 5C) were exclusively confined to the nuclear and cytosolic compartments, respectively.

The transfectants were also fixed and probed with Thr(P)-456 CAD antibodies. As expected, the micrographs showed moderate levels of phosphorylated CAD in the nucleus of cells transfected with pECFP-CAD. A much stronger fluorescent signal was observed localized within the nucleus of the pECFP-NLS-CAD transfectants. In contrast, only minimal levels of Thr(P)-456 CAD could be detected when CAD was excluded from the nucleus in the pECFP-NES-CAD transfectants.

The phosphorylation state of CAD in these three constructs was also assessed by immunoblotting. Although the level of expression of CAD was comparable in the three transfectants (Fig. 5A, upper panel), the immunoblot probed with CAD Thr(P)-456 antibodies (Fig. 5A, lower panel) confirmed that the extent of phosphorylation of NLS-CAD was higher than that in the wild type cells and that there was minimal phosphorylation of NES-CAD. The ratio of CAD Thr(P)-456 in NLS-CAD and NES-CAD compared with the wild type transfectant was 1.4 and 0.07, respectively.

**Physiological Consequences of Trapping CAD in the Cytosolic or Nuclear Compartments**—The rate of de novo pyrimidine biosynthesis was investigated by measuring the incorporation of the CPSase substrate, [14C]bicarbonate, into UTP and CTP. As expected, pyrimidine biosynthesis did not proceed at a detectable rate in the uridine auxotroph Urd A cells (Fig. 6A). Urd A cells stably transfected with wild type pECFP-CAD, pECFP-NLS-CAD, and pECFP-NES-CAD were used for these studies. The wild type CAD synthesized UTP at the rate of 2.43 pmol/min/10^6 cells (Fig. 6A), a rate comparable with that observed (14, 15) in BHK21 and MCF10A cells. The rate of incorporation of the radiolabel into CTP was the same as UTP when the lower intracellular steady state concentration of this nucleotide was taken into consideration (Fig. 6B). The activity of...
the pyrimidine biosynthetic pathway was slightly reduced to 2.35 pmol/min/10^6 cells in pECFP-NLS-CAD transfectants. In contrast, the activity of the pathway was reduced 22% to 1.9 pmol/min/10^6 cells in the pECFP-NES-CAD transfectants. The standard error of the measurement of flux through the pathway was found to be 5.9%. Thus, the small difference between the WT and pECFP-NLS-CAD transfectants may not be statistically significant, but the rate of pyrimidine biosynthesis in pECFP-NES-CAD cells was significantly lower than in the wild type pECFP-CAD cells.

The intracellular concentrations of UTP and CTP were found by HPLC analysis to be 0.10 and 0.02 mM, respectively, in the wild type transfectants, similar to the values reported for BHK cells (14) using the same method of analysis. The measurements of nucleotide pool size were found to be reproducible within 10%. The UTP and CTP pools in pECFP-NLS-CAD transfectants were reduced by 18% and a larger decrease, 33%, in the intracellular concentration of these nucleotides was found in the pECFP-NES-CAD transfectants.

The altered rate of pyrimidine biosynthesis and the smaller nucleotide pools were reflected in the growth rate of the cells. Whereas the growth of pECFP-NLS-CAD transfectants (Fig. 6C) was only slightly less than that of the pECFP-CAD transfectants, the growth rate of cells transfected with pECFP-NES-CAD was reduced by 60%.
Intracellular Localization of DHODhase and UMP Synthase—Human DHODhase and UMP synthase were cloned by PCR and inserted into mammalian expression vectors with an appended GFP tag. Immunofluorescence microscopy (Fig. 7A) of transiently transfected BHK21 cells showed that the DHODhase-GFP fusion protein was excluded from the nucleus and appeared to colocalize with the mitochondria. In contrast, GFP-UMP synthase (Fig. 7B), previously considered exclusively cytosolic (4), was found, like CAD, in both cytoplasmic and nuclear compartments.

DISCUSSION

When resting cells are induced to proliferate, the MAP kinase cascade is activated, and CAD Thr-456 is phosphorylated. Phosphorylation modulates the allosteric transitions, stimulating the CAD CPSase activity and increasing the flux through the pyrimidine biosynthetic pathway (5). Although the bulk of CAD in exponentially growing BHK21 cells, and five other cell lines tested, was soluble, much of the CAD Thr(P)-456 could be extracted from an insoluble fraction that was shown by cell fractionation and fluorescence microscopy to be derived from the nucleus.

The microscopic studies supported previous findings (17) showing that a part of cytosolic CAD was localized near the mitochondria. It is interesting that this colocalization was more pronounced for the fraction of Thr(P)-456 CAD found in the cytoplasm, suggesting that the activation of the protein may promote these interactions. CAD Thr-456 → Glu, a mutant designed to mimic phosphorylation, also colocalized to some extent with mitochondria (not shown). However, the interactions between the protein and this organelle must be weak or transient, because cell fractionation showed little CAD associated with the mitochondrial membranes or matrix.

The intracellular localization of CAD depended on the growth state. In resting cells, CAD was localized exclusively in the cytoplasm, but a significant fraction was imported into the nucleus upon stimulation by EGF. Analysis of the micrographs (Fig. 3 and Table IV) gave a ratio of CAD in the nucleus to cytoplasm of 0.2 in exponentially growing cells, indicating that the CAD concentration in the nucleus was 5-fold lower than that in the cytoplasm. In contrast, the distribution of CAD Thr(P)-456 was just the reverse with the level in the nucleus 2-fold higher than in the cytoplasm. Thus, there was a 10-fold difference in the distribution of CAD and Thr(P)-456 CAD in the cytoplasmic and nuclear compartments. The differential was appreciably larger when the nuclear and cytoplasmic subfractions were taken into consideration. Immunoblotting showed that the ratio of Thr(P)-456 CAD to unphosphorylated CAD in the insoluble nuclear fraction was 44-fold higher than that in the soluble cytoplasmic fraction.

CAD in the insoluble nuclear fraction was bound to nuclear substructures, including the nuclear matrix. Association of the protein with the nuclear matrix was observed previously in adenovirus-infected cells (18). The precursor to the preterminal protein, an essential component in viral replication, is anchored to the matrix by CAD at sites of active replication. Most interestingly, both CAD and the preterminal protein bound to the matrix are released upon incubation with ATP, an observation that the authors attributed to a Tyr-protein kinase-mediated phosphorylation (18) of one of the components in the complex. In accordance with these results, CAD associated with the nuclear matrix was also found to be partially released upon incubation with ATP. Large cellular DNA replication complexes are known to be associated in part with the nuclear matrix (40), and the participation of CAD in these complexes is an intriguing possibility. Besides the nuclear matrix, we cannot rule out interactions with other nuclear components, and the identification of other possible CAD-binding partners is currently under investigation.

The nuclear import of many proteins, including signaling molecules such as MAP kinase (32) and various transcription factors (33), is induced by the phosphorylation of specific residues in the translocated protein. The observation that the translocation of CAD into the nucleus coincides with phosphorylation of Thr-456 suggested that this modification might trigger the nuclear import that occurred upon stimulation of the cell by growth factors. However, two lines of evidence ruled out this possibility. 1) The MEK inhibitor PD98059 blocked activation of MAP kinase and CAD phosphorylation but did not have any effect on nuclear translocation. 2) Nuclear transloca-
tion proceeded normally in mutants in which Thr-456 was replaced by alanine or glutamate, indicating that MAP kinase phosphorylation, essential for the activation of the pathway, does not affect nucleocytoplasmic dynamics. The signals that trigger nuclear import of CAD are unknown but are now under active investigation.

Constructs were engineered in which CAD was restricted to either the nuclear or cytoplasmic compartments. NLS-CAD was confined exclusively to the nucleus, and its MAP kinase site was extensively phosphorylated, suggesting that phosphorylation may occur within the nucleus. This interpretation is reasonable because the bulk of the activated MAP kinase is known to be localized in the nucleus (34). However, we cannot rule out the possibility that CAD is rapidly phosphorylated in the cytoplasm prior to nuclear importation. Conversely, there was little Thr(P)-456 CAD in the cytoplasm of the NES transfectants, 0.7% compared with wild type CAD, suggesting that confining CAD to the cytoplasmic compartment interferes with MAP kinase phosphorylation. This observation is consistent with the interpretation that CAD phosphorylation occurs primarily in the nucleus, although we cannot rule out the possibility that phosphatases rapidly dephosphorylate cytoplasmic CAD. The latter interpretation is less likely because the cytoplasmic levels of Thr(P)-456 CAD in the wild type transfectants were significantly higher than that observed for NLS-CAD. Thus, although alternative arguments could be made, collectively these experiments suggest that phosphorylation of CAD in the cytoplasm is minimal and that it is translocated into the nucleus by a MAP kinase-independent mechanism and then phosphorylated on Thr-456.

The question then becomes, does nuclear CAD participate in pyrimidine biosynthesis? A potential disadvantage of localizing the pyrimidine biosynthetic enzymes in different cellular compartments results from the reversibility of the reaction catalyzed by DHOase. At physiological pH, the equilibrium ratio of carbamoyl aspartate to dihydroorotate is 20 to 1 (35). Thus, the major product of CAD is carbamoyl aspartate and not the end product dihydroorotate. To maintain forward flux through the pathway, CAD must be coupled to the next enzyme, mitochondrial DHOdhase. It is known for example that partial channeling of carbamoyl aspartate between ATPase and DHOase occurs only in the presence of DHOdhase (36).

The effect of confining CAD exclusively to one or the other cellular compartments on the rate of pyrimidine biosynthesis, the pyrimidine nucleotide pools, and the growth rate of the cells was examined using pECFP-CAD, pECFP-NLS-CAD, and pECFP-NES-CAD Urd A transfectants. The activity of the pyrimidine pathway was nearly the same in pECFP-NLS-CAD transfecteds as in the wild type cells, although there were small reductions in nucleotide pool sizes and growth rate. This result suggests that dihydroorotate synthesized in the nucleus can be used nearly as efficiently by mitochondrial DHOdhase as the wild type transfectants. In contrast, confining CAD to the cytoplasm reduced the rate of pyrimidine biosynthesis and the UTP and CTP pools and led to a significant reduction in the cytoplasmic compartment. UTP synthase has been considered a cytosolic protein, so the final two steps of the pathway occur solely in the cytosol (4). However, fluorescence microscopy revealed its presence in both the cytoplasm and nucleus. Thus, it is possible when CAD is activated during the S phase of the cell cycle that five of the six steps of the pathway occur within the nucleus and the sixth step takes place in the proximal mitochondria (Fig. 7C). Although somewhat controversial (38), there is evidence for distinct nuclear and cytosolic nucleotide pools that exchange slowly. In this event, if the end product of the pathway is synthesized in the nucleus, it would be strategically located to provide precursors to replenish the deoxyribonucleotide pools.

In summary, pyrimidine biosynthesis in resting cells occurs in the cytoplasmic compartment where interactions between CAD and the mitochondria may facilitate the oxidation of dihydroorotate. When induced to proliferate, a fraction of the cytosolic CAD is both translocated into the nucleus and activated by MAP kinase phosphorylation. A plausible hypothesis is that MAP kinase phosphorylation of CAD occurs primarily within the nucleus, but in any event MAP kinase phosphorylation was not a prerequisite for nuclear import. Pyrimidine biosynthesis and cell growth proceeded apace in mutants that confined CAD to the nucleus but were reduced when the protein was restricted to the cytoplasmic compartment. Thus, it is likely that nuclear import facilitates optimal growth of the cell.

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2 Although NES-CAD was likely to be translocated into the nucleus, the lack of significant phosphorylation may be attributed to the strong nuclear export signals incorporated into the molecule so that it did not reside within this organelle long enough to allow CAD phosphorylation.
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