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The human Rad9 checkpoint protein stimulates the carbamoyl phosphate synthetase activity of the multifunctional protein CAD

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ABSTRACT

The human Rad9 checkpoint protein is a subunit of the heterotrimeric Rad9-Rad1-Hus1 (9-1-1) complex that plays a role as a damage sensor in the DNA damage checkpoint response. Rad9 has been found to interact with several other proteins outside the context of the 9-1-1 complex with no obvious checkpoint functions. During our studies on the 9-1-1 complex, we found that Rad9 immunoprecipitates contained a 240 kDa protein that was identified as carbamoyl phosphate synthetase/aspartate transcarbamoylase/dihydroorotase (CAD), a multienzymatic protein required for the *de novo* synthesis of pyrimidine nucleotides and cell growth. Further investigations revealed that only free Rad9, but not Rad9 within the 9-1-1 complex, bound to CAD. The rate-limiting step in *de novo* pyrimidine nucleotide synthesis is catalyzed by the carbamoyl phosphate synthetase II (CPSase) domain of CAD. We find that Rad9 binds to the CPSase domain, and, moreover, this binding results in a 2-fold stimulation of the CPSase activity of CAD. Similar results were also obtained with an N-terminal Rad9 fragment. These findings suggest that Rad9 may play a role in ribonucleotide biosynthesis.

INTRODUCTION

The human Rad9, Rad1 and Hus1 proteins make a proliferating cell nuclear antigen (PCNA)-like heterotrimeric complex that has a ring structure and is thought to play a PCNA-like role as a DNA clamp specific for the DNA damage checkpoint response [reviewed in (1)]. It has been found that the checkpoint-specific Rad17-replication factor C (RFC) specifically binds to the Rad9-Rad1-Hus1 (9-1-1) complex and clamps it around a DNA duplex (2–5). Although the precise mechanism by which the Rad17-RFC/9-1-1 complex clamp-loader/checkpoint-clamp senses damage and initiates cell cycle arrest is not known, significant progress has been made in understanding the role of this complex.

Investigations into how the 9-1-1 complex may transduce a signal to downstream proteins in the checkpoint response have revealed that subunits of the complex, in particular Rad9, interact with a number of other cellular proteins with no obvious role in cell cycle arrest and have raised the possibility that these proteins might individually participate in other cellular functions as well. For example, Rad9 has recently been shown to bind to the androgen receptor and modulate its activity (6). Rad9 also binds to the kinases c-Abl (7) and PKCδ (8), which in turn regulate the binding of Rad9 to the antiapoptotic proteins Bcl-2 and Bcl-x_L, suggesting that Rad9 may play a role in the apoptotic response to genotoxic stress (9).

In the course of our studies on the function of the 9-1-1 complex, we found that when Rad9 was purified by immunoaffinity chromatography from extracts of transiently transfected human cells, the purified Rad9 preparation contained a protein of 240 kDa as a major contaminant. Mass spectrometric analysis was used to identify this protein as carbamoyl phosphate synthetase/aspartate transcarbamoylase/dihydroorotase (CAD), a multienzymatic protein that catalyzes the first three steps in *de novo* pyrimidine synthesis (10). The rate-limiting step in this pyrimidine synthesis pathway is catalyzed by the carbamoyl phosphate synthetase II (CPSase) of CAD, which is also the site of feedback inhibition by uridine nucleotides and activation by the allosteric ligand phosphoribosyl pyrophosphate (PRPP) (11). CPSase activity is increased in tumor cells (12,13) and is regulated by the protein kinase A and mitogen-activated protein kinase signaling pathways in a growth- and cell-cycle-dependent manner (14–17). We find that Rad9 binds to the CPSase domain and that this binding results in a 2-fold stimulation of the CPSase activity of CAD. This is the first documented report of a protein–protein interaction with CAD regulating its CPSase activity. We find that CAD interacts with free Rad9, but not Rad9 within the 9-1-1 checkpoint complex. These findings suggest that Rad9 may have an additional role to its checkpoint function in ribonucleotide biosynthesis.

MATERIALS AND METHODS

Materials

Antisera that recognize various antigens were obtained as follows: rabbit anti-His_6 (H-15) and rabbit anti-hRad9

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(M-389) from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-Flag from Sigma (St Louis, MO).

Transfections and immunoprecipitations

Human embryonic kidney 293T cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum, 100 U of penicillin and streptomycin per milliliter and were transfected with pcDNA3-FlaghRad9, pcDNA3-His_6FlagCAD, pcDNA3-His_6-FlagCPSII or pcDNA3-HA-hRad9 plasmids using the calcium phosphate transfection method as described previously (18). Forty-eight hours after transfection, cells were washed with phosphate-buffered saline (PBS) and lysed in 20 packed cell volumes of Lysis Buffer [50 mM Tris–HCl (pH 7.5), 0.3 M NaCl, 0.5% Nonidet P-40 (NP-40), protease inhibitors (Roche Molecular Biochemicals)]. After incubating 15 min on ice, the cell lysate was centrifuged for 30 min at 32 000 *g*. The supernatant was incubated with anti-Flag agarose (Sigma) (20 µl/ml of lysate) for 4 h at 4°C. The resin was then washed twice with Lysis Buffer, twice with Tris-buffered saline (TBS) [50 mM Tris–HCl (pH 7.5), 150 mM NaCl] and the protein was eluted for 30 min at 4°C with TBS containing 0.2 mg/ml Flag peptide (Sigma).

Expression and purification of recombinant proteins

Baculoviruses were described previously for expression of full-length Flag-Rad9 (18), fragments of Rad9 (2,18) and His_6-CAD (19). A monolayer of 2×10^8 High Five insect cells (Invitrogen) were infected with a multiplicity of infection of five with either the His_6-CAD baculovirus alone or together with the Flag-Rad9 baculovirus and then harvested after 48 h. The cells were washed with PBS and lysed in 20 packed cell volumes of lysis buffer [50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 15 mM imidazole, 0.5% Nonidet P-40 (NP40) and protease inhibitors]. After incubating 15 min on ice, the cell lysate was centrifuged for 30 min at 32 000 *g*. The supernatant was incubated with Ni-NTA agarose (Qiagen) (50 µl/ml of lysate) for 4 h at 4°C. The resin was then washed twice with lysis buffer, twice with elution buffer [50 mM Tris–HCl (pH 7.5), 10% glycerol, 0.3 M NaCl, 2 mM ATP] and then eluted with two volumes of elution buffer containing 100 mM imidazole. Typical protein yields of 30 µg were obtained.

Interactions between CAD and Rad9, 9-1-1 and Rad9 fragments

H5 insect cells were co-infected with baculoviruses expressing either His-CAD alone or together with either Flag-Rad9, or Flag-Hus1 and His-Rad1, or Flag-Hus1 and His-Rad1 and untagged Rad9, or the five fragments of Rad9 which were all described previously (2). After lysing the cells in 50 mM Tris–HCl (pH 7.5), 0.3 M NaCl, 0.5% NP40, the protein was then immunoaffinity purified using anti-Flag agarose. After washing the resin three times in the same buffer, the protein was eluted with Flag peptide and analyzed by western blotting.

CPSase activity assay

CPSase activity was measured essentially as described previously (19). Briefly, all of the reaction components except the cold and ^{14}C-labeled sodium bicarbonate (the initiation solution) were added to tubes on ice. These samples then were placed in a 37°C water bath for 10 min before the initiation mix was added to start the reaction. The 250 µl reaction was allowed to proceed for 30 min at 37°C before it was quenched by the addition of 125 µl of 80% trichloroacetic acid. The unincorporated ^{14}C was then removed by gently heating the samples at 85°C for 2–3 h. The incorporation of ^{14}C into the acid stable carbamoyl aspartate was then measured by scintillation counting. The reaction mixture, contained 7.5 µl of the elution buffer previously described, containing ~0.11 µg of CAD, 87 mM Tris–HCl (pH 8.0), 87 mM KCl, 6.5% dimethyl sulfoxide, 2.2% glycerol, 0.87 mM dithiothreitol, 3.1 mM glutamine, 17.4 mM aspartate, 7 mM [^{14}C]$NaHCO_3$ (50 mCi/mmol), 2 mM excess of $MgCl_2$ over the addition of nucleotide and PRPP concentrations and ATP concentrations (adjusted to ATP contained within the protein elution buffer) varying from 0.06 to 15 mM.

RESULTS

Copurification of CAD with Rad9

In the course of our studies on the 9-1-1 complex, we found that when HEK293T cells were transfected with a plasmid expressing Flag-tagged Rad9 and the protein was purified with anti-Flag agarose, the preparation contained a major band of 240 kDa and a minor band of 110 kDa (Figure 1). The preparation also contained two bands ~30 kDa consistent with the sizes of Rad1 and Hus1, which were at levels stoichiometrically similar to the 240 kDa band when analyzed by coomassie staining (data not shown). None of these bands were seen in immunoaffinity-purified extracts from control cells transfected with the vector alone. Mass spectroscopic analysis of Rad9-copurifying bands revealed that the

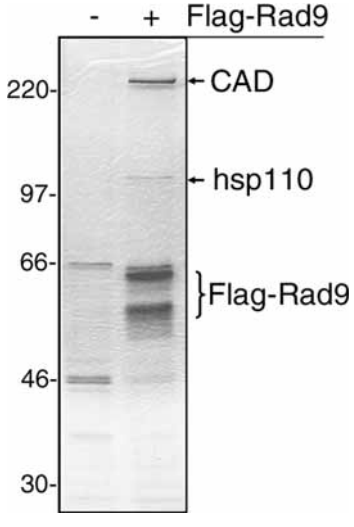

Figure 1. Copurification of CAD with Rad9. An aliquot of 3×10^6 293T cells were mock treated (−) or transfected with 6 µg pcDNA3-Flag-hRad9 (+). Protein was immunoprecipitated using anti-Flag agarose from whole-cell extracts, and 10% of the protein eluted with Flag peptide was visualized after SDS–PAGE by silver staining. Mass spectrometric analysis was performed by the University of North Carolina Mass Spectrometry Core Facility.

240 kDa protein is CAD and the 110 kDa protein is the heat-shock protein hsp110. As heat-shock proteins often copurify with ectopically expressed proteins, we concentrated our efforts on characterizing CAD, the major protein that copurifies with Rad9.

Mapping of CAD and Rad9 domains required for binding

The human CAD protein catalyzes three separate reactions with three distinct structural domains, CPSase, aspartate transcarbamoylase (ATCase) and dihydroorotase (DHOase) (Figure 2A). Similarly, human Rad9 has an N-terminal PCNA-like domain and a C-terminal extension which is heavily phosphorylated and is thought to play a role as an effector in checkpoint signaling (20–22) (Figure 2B). To gain insight into the potential physiological significance of the CAD–Rad9 interaction, we mapped the interacting regions on both proteins by deletion analysis. In Figure 3, both full-length CAD and the CPSase domain of CAD were co-expressed with Rad9 in 293T cells and the CAD and CPSase immunoprecipitates were immunoblotted for Rad9. As seen in Figure 3 (right panel), Rad9 bound equally well to the full-length CAD and to the CPSase domain alone. To perform reciprocal experiments, insect cells were infected with baculoviruses expressing CAD together with regions of Rad9. The Rad9 fragments were immunoprecipitated and the precipitates were analyzed for CAD by immunoblotting. As seen in Figure 4, the N-terminal and the middle third, but not the C-terminal third of Rad9 bound to CAD. The strongest binding was observed with the N-terminal two-thirds of the protein. Interestingly, the C-terminal two-thirds bound CAD with less affinity than the middle third alone, suggesting that the C-terminal tail may negatively regulate the Rad9–CAD interaction. While the quantitative aspects of the data regarding the binding strength has certain limitations, this data unambiguously show that the PCNA-like domain of Rad9 binds to CAD whereas the C-terminal domain, which encompasses the major Rad9 phosphorylation sites and presumably acts as an effector in checkpoint signaling, does not.

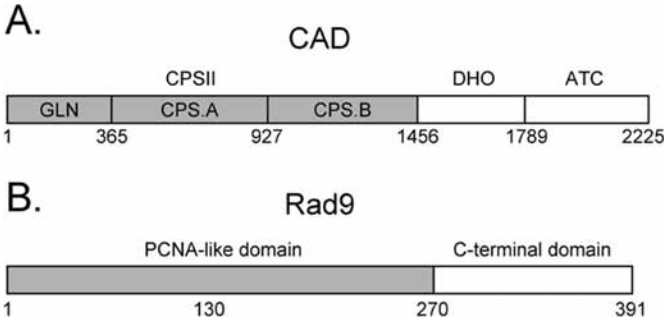

Figure 2. Structural organization of CAD and Rad9. (**A**) The human CAD protein has three distinct structural domains performing the three enzymatic activities, carbamoyl phosphate synthetase (CPS II), aspartate transcarbamoylase (ATC) and dihydroorotase (DHO). The CPS II domain is indicated with gray shading. (**B**) Human Rad9 has an N-terminal PCNA-like domain (gray shading) and a heavily phosphorylated C-terminal domain.

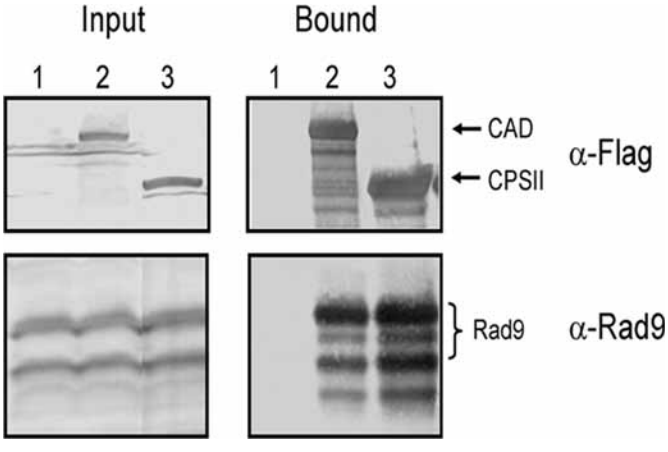

Figure 3. Rad9 interacts with the CPSII domain of CAD. An aliquot of 3×10^6 293T cells was transfected with 6 μg of pcDNA3-HA-Rad9 alone (lane 1) or along with 6 μg of pcDNA3-Flag-CAD (lane 2), or pcDNA3-Flag-CPSII (lane 3). The proteins were immunoprecipitated with anti-Flag agarose and then analyzed by western blotting with anti-Rad9 and anti-Flag antibodies as indicated.

CAD binds to free Rad9 but not to Rad9 within the 9-1-1 complex

To investigate whether CAD may participate in Rad17-RFC/9-1-1 complex-mediated signal transduction, we tested the binding of CAD to the other members of the 9-1-1 complex,

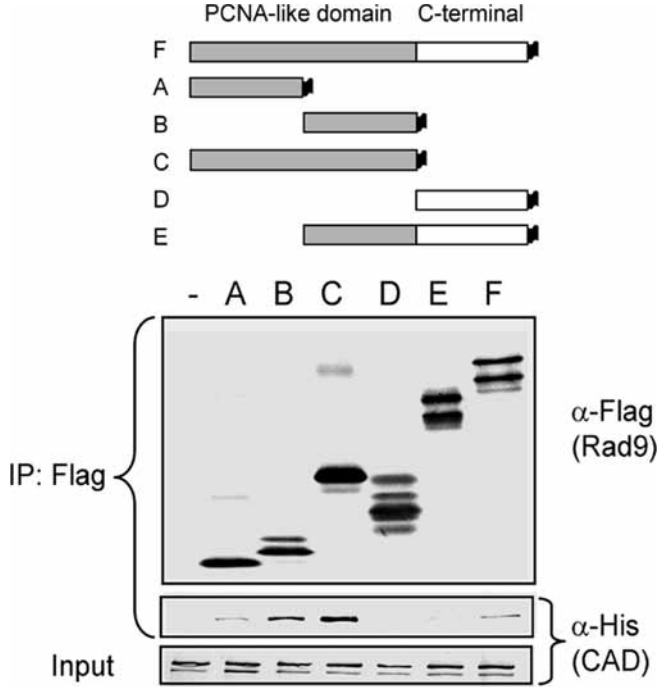

Figure 4. CAD interacts with the PCNA-like domain of Rad9. Insect cells were infected with His-CAD alone (−) or together with Flag-tagged fragments of Rad9 (fragment A contains amino acids 1–130, fragment B contains amino acids 130–270, fragment C contains amino acids 1–270, fragment D contains amino acids 260–391 and fragment E contains amino acids 130–391) or with full-length Rad9 (F). The proteins were immunoprecipitated with anti-Flag agarose and then analyzed by western blotting with anti-His and anti-Flag antibodies as indicated.

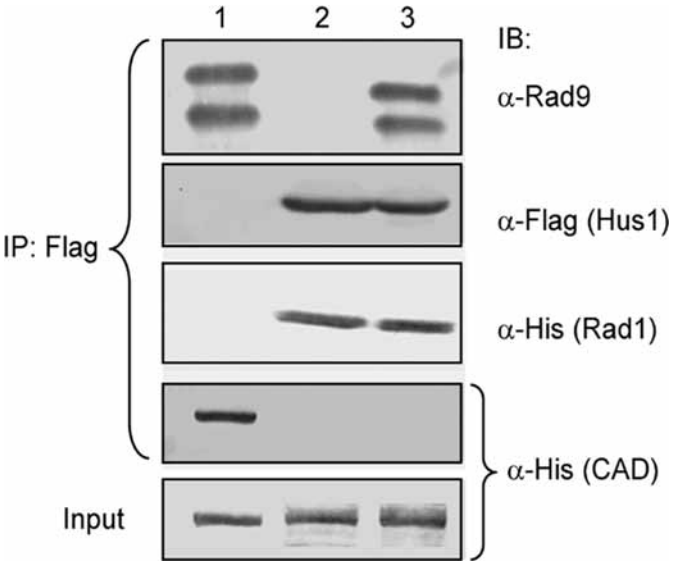

Figure 5. CAD binds to free Rad9 but not to Rad9 within the 9-1-1 complex. Insect cells were co-infected with His-CAD together with Flag-Rad9 (lane 1), or Flag-Hus1 and His-Rad1 (lane 2), or Flag-Hus1, His-Rad1 and untagged-Rad9 (lane 3). The proteins were immunoprecipitated with anti-Flag agarose and then analyzed by western blotting with anti-His, anti-Rad9 and anti-Flag antibodies as indicated.

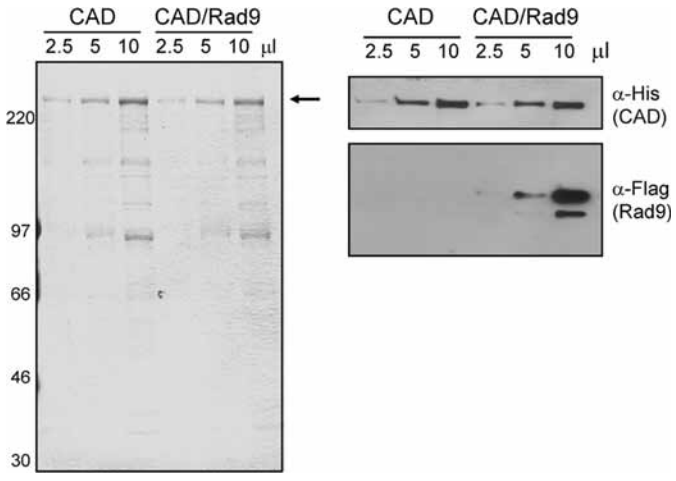

Figure 6. Purification of CAD with and without Rad9. Insect cells were infected with His-CAD alone or together with Flag-Rad9. The proteins were affinity purified with Ni-agarose and then analyzed by Coomassie staining (left panel) and western blotting (right panels). The arrow indicates the position of CAD. Aliquots of 2.5, 5, 10 μl from each sample was loaded which correlates to ~50, 100, 200 ng of CAD and 25, 50, 100 ng of Rad9 when quantitated using protein standards in the same gel.

Rad1 and Hus1, as well as to the ternary 9-1-1 complex by co-infection of insect cells with combinations of baculoviruses that express these proteins. The tagged 9-1-1 proteins were immunoprecipitated and the immunoprecipitates were tested for the other 9-1-1 proteins and for CAD. Figure 5 shows that Rad9 expressed in insect cells, such as Rad9 expressed in mammalian cells, binds CAD, but Rad1 and Hus1 do not (lane 1 versus lane 2). Most significantly, when Rad9 is co-immunoprecipitated as part of the 9-1-1 complex by using a Hus1 affinity tag, the immunoprecipitate does not contain any detectable CAD (lane 3). This was somewhat expected because Rad9 interacts with Rad1 and Hus1 through its PCNA-like domain suggesting that this domain is apparently blocked from binding to CAD within the 9-1-1 complex. These findings suggested that the Rad9–CAD interaction may have a role in cellular physiology other than DNA damage checkpoint signaling.

Modulation of carbamoyl phosphate synthetase activity

Since currently there is no specific assay for Rad9 activity, we tested for the possible modulation of CAD activity by Rad9 in the following series of experiments. Initially, we found that the immunoaffinity-purified Rad9–CAD complex exhibited higher CPSase activity than CAD alone (data not shown). To further examine the effect of Rad9 on CAD activity, we examined whether purified proteins could be combined to recapitulate this regulation. However, when combined *in vitro*, the two proteins did not form a stable complex, and we observed no change in the CPSase activity of CAD (data not shown). We reasoned that the two proteins may have to be co-expressed to form a complex, and therefore we expressed CAD alone and CAD plus Rad9 in insect cells and purified these recombinant proteins using the His_6 tag on CAD for affinity purification. We obtained the protein preparations shown in Figure 6. The CAD protein levels obtained under the two conditions were quite similar, and the amount of CAD activity was normalized to the amount of CAD protein in each sample as determined by quantitative western analysis.

CPSase synthesizes carbamoyl phosphate from ATP, bicarbonate and glutamine, and is the first enzymatic step in pyrimidine biosynthesis. CPSase is allosterically activated by PRPP and inhibited by UTP (10). We tested the effect of Rad9 on CPSase activity in the presence of saturating amounts of PRPP with varying ATP concentrations. We observed that the V_{max} of CAD was increased 2-fold by Rad9 both in the presence or absence of PRPP (Figure 7 and Table 1). Kinetic analysis of CAD/Rad9 complexes revealed that Rad9 did not significantly change the apparent affinity for ATP (K_m) in the presence or absence of PRPP. Likewise, a fragment of Rad9 lacking the C-terminal phosphorylation sites (fragment C in Figure 4) had a similar effect on the CPSase activity of CAD (Figure 7 and Table 1). Thus, it appears that Rad9 regulates the rate-limiting step of ribonucleotide biosynthesis by increasing the V_{max} of the CPSase activity.

DISCUSSION

Transcriptional induction of ribonucleotide reductase (RNR) is one of the best-characterized transcriptional response reactions to DNA damage and has been observed in organisms ranging from *Escherichia coli* to humans (23). The upregulation of RNR activity by either transcriptional or post-transcriptional means increases the dNTP pools, and it has been shown that an increase in dNTP levels following DNA damage increases cellular survival in budding yeast (24). Since rNTP pools are typically 100-fold higher than the dNTP pools, it has generally been assumed that changes in rNTP levels would not affect the dNTP pool and hence the survival

and mutation rate. This assumption, however, has not been experimentally tested. Furthermore, RNA synthesis which would be affected by rNTP levels might contribute to cellular recovery from genotoxic stress. Such an outcome of a Rad9–CAD interaction would place this interaction within the general DNA damage response network. However, it is also plausible that the Rad9–CAD interaction is operational under physiological conditions to maintain cellular homeostasis and that this function is not related to the checkpoint function of Rad9 which occurs within the context of the 9-1-1 complex. Indeed, Rad9 has been found to associate with a number of proteins with diverse functions some of which are not related the DNA damage checkpoint response. Additionally, *de novo* pyrimidine biosynthesis is not only important in dividing cells for the production of new nucleic acids, but also for UDP–sugars and CDP–lipids, which are used for diverse cellular functions [reviewed in (17)]. Therefore, the Rad9-induced increase in CAD activity could be important in many different areas of cell physiology.

We currently do not know the cellular location of the CAD–Rad9 interaction. While CAD is mainly cytoplasmic, some studies have provided evidence for a fraction of CAD being in the nucleus (16,25,26). Under normal cellular conditions, Rad9 is part of the nuclear 9-1-1 complex (27,28). However, Rad9 does appear to have an independent cytoplasmic function during apoptosis. It has been demonstrated that during apoptosis Rad9 is cleaved by caspase-3 and the N-terminal portion of Rad9 translocates to the cytosol where it interacts with Bcl-x_L (29). Since CAD does not associate with the 9-1-1 complex (Figure 5), and all detectable nuclear Rad9 is in this complex (27), it is likely that the CAD–Rad9 interaction takes place in the cytoplasm. We could not detect the Rad9–CAD interaction by immunoprecipitating endogenous Rad9 presumably because the interaction is weak relative to the 9-1-1 interaction and therefore is detectable only when Rad9 is overproduced.

CAD is a unique biosynthetic enzyme with three distinct enzymatic activities. The CPSase enzyme domain catalyzes

Table 1. Kinetic parameters of wild-type CAD and CAD co-expressed with Rad9 and Rad9 fragment C

	Ligand	K_m	V_{max}
CAD	None	4.5 ± 0.43	7.3 ± 0.38
	PRPP	0.4 ± 0.05	13.4 ± 0.43
CAD/Rad9	None	4.6 ± 0.40	17.8 ± 0.86
	PRPP	0.5 ± 0.07	26.8 ± 0.99
CAD/Rad9 fragment C	None	3.8 ± 0.40	14.3 ± 0.65
	PRPP	0.5 ± 0.10	27.3 ± 1.67

The kinetic parameters of the CPSase data from CAD and CAD/Rad9 represent pooled data from five separate experiments, while the data from the CAD/Rad9 fragment represents pooled data from two separate experiments. K_m (mM ATP) is the apparent affinity for ATP, and V_{max} (pmol min^{-1}) is the maximum CPSase velocity measured as a function of ATP concentration. These data are the numerical results from the graphs in Figure 7.

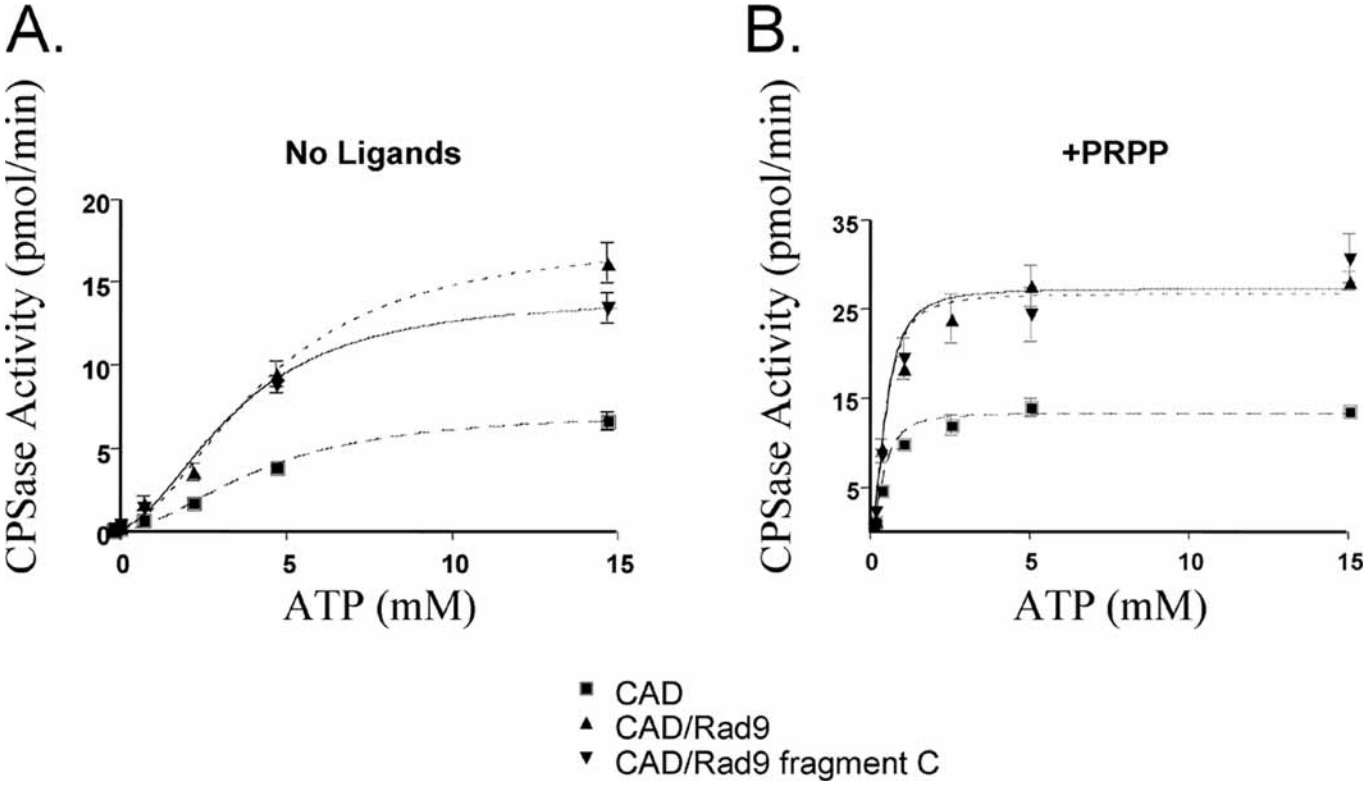

Figure 7. Rad9 increases the CPSase V_{max}. CPSase activity was measured from purified CAD (square) expressed alone or co-expressed with full-length Rad9 (triangle) or Rad9 with a C-terminal deletion (upside-down triangle) in the absence (**A**) or presence (**B**) of 2 mM PRPP with varying concentrations of ATP. The CAD and CAD/Rad9 data represent pooled results from five separate experiments, while the data from the CAD/Rad9 fragment C represent pooled data from two separate experiments. The curves were generated using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com) by performing nonlinear regression analysis to fit the raw data to the equation $Y = V_{max} * X^h/(K^h + X^h)$ where h is the Hill coefficient (set to 2) and K is the apparent K_m.

the formation of carbamoyl phosphate from glutamine, bicarbonate and two molecules of ATP. The ATCase region catalyzes the synthesis of carbamoyl aspartate from aspartate and carbamoyl phosphate. Finally, the formation of dihydroorotate is catalyzed by the DHOase domain (11). The assay used in these studies to measure CPSase activity is a coupled enzyme assay which measures the incorporation of [^{14}C] into the acid-stable chemical carbamoyl aspartate, which, as mentioned above, is catalyzed by the enzymatic activities of both the CPS II and ATC domains. However, since the CPSase activity is rate limiting (10,30), the observed changes in enzymatic activity are most likely due to changes in the CPSase activity of CAD.

The CPSase activity of CAD is highly regulated. As mentioned previously, it is allosterically activated by PRPP and inhibited by the product of pyrimidine-nucleotide synthesis, uridine 5′-triphosphate (UTP). Moreover, phosphorylation can affect the allosteric regulation by PRPP and UTP (14,31). An additional level of regulation of CPSase activity is by protein degradation induced by caspase-3 (19). The findings reported in this study add a previously unknown mode of CPSase activity regulation. Our data reveal the first documented report that a protein–protein interaction with CAD can regulate CPSase activity. Although allosteric regulators (i.e. PRPP and UTP) decrease the K_m for a CPSase substrate (ATP), our data suggests that Rad9 increases the V_{max} of CPSase rather than changing the K_m for ATP. Rad9 binding was also observed to increase the maximum velocity of CPSase activity in the presence of PRPP, but did not affect the percentage activation by PRPP. We investigated the affect of Rad9 on UTP-induced inhibition of CPSase activity. Rad9 did not significantly decrease the inhibitory action of UTP, but this result varied between CAD protein preparations preventing us from making a conclusion from this data (data not shown).

The mechanism by which Rad9 increases CPSase activity is not known. Rad9 binding could increase the affinity of other CPS II substrates such as glutamine or bicarbonate. Another plausible hypothesis is that Rad9 increases the glutaminase activity of CPS II, thereby increasing the overall V_{max} without affecting the affinity for ATP. However, this appears unlikely since experiments using ammonia as the nitrogen donor, thereby bypassing the need for the glutaminase activity, have shown that Rad9 still induced a 2-fold increase in CPSase activity (data not shown). Alternatively, Rad9 binding may induce a conformational shift that affects the oligomeric (activated) form of the enzyme. Active CAD has been shown to be a hexamer (31,32), and Rad9 may increase CAD activity by increasing the amount of enzyme in the hexameric form. Dissociation of CAD to monomers did not affect the CPSase activity, but significantly reduced the V_{max} of ATCase (32). It is therefore possible that Rad9 could be increasing CAD activity by stabilizing the oligomeric form of this enzyme and thereby increasing the V_{max} of ATCase. However, this seems unlikely since the CPSase activity of CAD is the rate-limiting step (30), and an increase in the ATCase rate would not be expected to increase the overall rate of our coupled enzyme reaction.

In summary, we find that the checkpoint protein Rad9 specifically interacts with the CPSase domain of CAD and increases its V_{max} by at least a factor of two. The high specificity of the Rad9–CAD interaction strongly suggests that it is of significance to normal cellular homeostasis. Further work is needed to determine the potential role of this interaction in regulating DNA damage checkpoints, ribonucleotide pools or RNA and DNA synthesis.

ACKNOWLEDGEMENTS

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REFERENCES

1. Sancar,A., Lindsey-Boltz,L.A., Unsal-Kacmaz,K. and Linn,S. (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu. Rev. Biochem.*, **73**, 39–85.
2. Bermudez,V.P., Lindsey-Boltz,L.A., Cesare,A.J., Maniwa,Y., Griffith,J.D., Hurwitz,J. and Sancar,A. (2003) Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex *in vitro*. *Proc. Natl Acad. Sci. USA*, **100**, 1633–1638.
3. Zou,L., Liu,D. and Elledge,S.J. (2003) Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc. Natl Acad. Sci. USA*, **100**, 13827–13832.
4. Ellison,V. and Stillman,B. (2003) Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5′ recessed DNA. *PLoS Biol.*, **1**, E33.
5. Majka,J. and Burgers,P.M. (2003) Yeast Rad17/Mec3/Ddc1: a sliding clamp for the DNA damage checkpoint. *Proc. Natl Acad. Sci. USA*, **100**, 2249–2254.
6. Wang,L., Hsu,C.-L., Ni,J., Wang,P.-H., Yeh,S., Keng,P. and Chang,C. (2004) Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells. *Mol. Cell. Biol.*, **24**, 2202–2213.
7. Yoshida,K., Komatsu,K., Wang,H.G. and Kufe,D. (2002) c-Abl tyrosine kinase regulates the human Rad9 checkpoint protein in response to DNA damage. *Mol. Cell. Biol.*, **22**, 3292–3300.
8. Yoshida,K., Wang,H.G., Miki,Y. and Kufe,D. (2003) Protein kinase C delta is responsible for constitutive and DNA damage-induced phosphorylation of Rad9. *EMBO J.*, **22**, 1431–1441.
9. Komatsu,K., Miyashita,T., Hang,H., Hopkins,K.M., Zheng,W., Cuddeback,S., Yamada,M., Lieberman,H.B. and Wang,H.G. (2000) Human homologue of *S. pombe* Rad9 interacts with BCL-2/BCL-xL and promotes apoptosis. *Nature Cell Biol.*, **2**, 1–6.
10. Jones,M.E. (1980) Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis. *Annu. Rev. Biochem.*, **49**, 253–279.
11. Carrey,E.A. and Hardie,D.G. (1988) Mapping of catalytic domains and phosphorylation sites in the multifunctional pyrimidine-biosynthetic protein CAD. *Eur. J. Biochem.*, **171**, 583–588.
12. Reardon,M.A. and Weber,G. (1985) Increased carbamoyl-phosphate synthetase II concentration in rat hepatomas: immunological evidence. *Cancer Res.*, **45**, 4412–4415.
13. Sigoillot,F.D., Sigoillot,S.M. and Guy,H.I. (2004) Breakdown of the regulatory control of pyrimidine biosynthesis in human breast cancer cells. *Int. J. Cancer*, **109**, 491–498.
14. Graves,L.M., Guy,H.I., Kozlowski,P., Huang,M., Lazarowski,E., Pope,R.M., Collins,M.A., Dahlstrand,E.N., Earp,H.S.,III and Evans,D.R. (2000) Regulation of carbamoyl phosphate synthetase by MAP kinase. *Nature*, **403**, 328–332.
15. Sigoillot,F.D., Evans,D.R. and Guy,H.I. (2002) Growth-dependent regulation of mammalian pyrimidine biosynthesis by the protein kinase A and MAPK signaling cascades. *J. Biol. Chem.*, **277**, 15745–15751.
16. Sigoillot,F.D., Berkowski,J.A., Sigoillot,S.M., Kotsis,D.H. and Guy,H.I. (2003) Cell cycle-dependent regulation of pyrimidine biosynthesis. *J. Biol. Chem.*, **278**, 3403–3409.
17. Huang,M. and Graves,L.M. (2003) De novo synthesis of pyrimidine nucleotides; emerging interfaces with signal transduction pathways. *Cell. Mol. Life Sci.*, **60**, 321–336.

18. Lindsey-Boltz,L.A., Bermudez,V.P., Hurwitz,J. and Sancar,A. (2001) Purification and characterization of human DNA damage checkpoint Rad complexes. *Proc. Natl Acad. Sci. USA*, **98**, 11236–11241.
19. Huang,M., Kozlowski,P., Collins,M., Wang,Y., Haystead,T.A. and Graves,L.M. (2002) Caspase-dependent cleavage of carbamoyl phosphate synthetase II during apoptosis. *Mol. Pharmacol.*, **61**, 569–577.
20. Roos-Mattjus,P., Hopkins,K.M., Oestreich,A.J., Vroman,B.T., Johnson,K.L., Naylor,S., Lieberman,H.B. and Karnitz,L.M. (2003) Phosphorylation of human Rad9 is required for genotoxin-activated checkpoint signaling. *J. Biol. Chem.*, **278**, 24428–24437.
21. St Onge,R.P., Besley,B.D., Park,M., Casselman,R. and Davey,S. (2001) DNA damage-dependent and -independent phosphorylation of the hRad9 checkpoint protein. *J. Biol. Chem.*, **276**, 41898–41905.
22. St Onge,R.P., Besley,B.D., Pelley,J.L. and Davey,S. (2003) A role for the phosphorylation of hRad9 in checkpoint signaling. *J. Biol. Chem.*, **278**, 26620–26628.
23. Elledge,S.J., Zhou,Z., Allen,J.B. and Navas,T.A. (1993) DNA damage and cell cycle regulation of ribonucleotide reductase. *Bioessays*, **15**, 333–339.
24. Chabes,A., Georgieva,B., Domkin,V., Zhao,X., Rothstein,R. and Thelander,L. (2003) Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. *Cell*, **112**, 391–401.
25. Carrey,E.A., Dietz,C., Glubb,D.M., Loffler,M., Lucocq,J.M. and Watson,P.F. (2002) Detection and location of the enzymes of de novo pyrimidine biosynthesis in mammalian spermatozoa. *Reproduction*, **123**, 757–768.
26. Angeletti,P.C. and Engler,J.A. (1998) Adenovirus preterminal protein binds to the CAD enzyme at active sites of viral DNA replication on the nuclear matrix. *J. Virol.*, **72**, 2896–2904.
27. Burtelow,M.A., Roos-Mattjus,P.M., Rauen,M., Babendure,J.R. and Karnitz,L.M. (2001) Reconstitution and molecular analysis of the hRad9-hHus1-hRad1 (9-1-1) DNA damage responsive checkpoint complex. *J. Biol. Chem.*, **276**, 25903–25909.
28. Burtelow,M.A., Kaufmann,S.H. and Karnitz,L.M. (2000) Retention of the human Rad9 checkpoint complex in extraction-resistant nuclear complexes after DNA damage. *J. Biol. Chem.*, **275**, 26343–26348.
29. Lee,M.W., Hirai,I. and Wang,H.G. (2003) Caspase-3-mediated cleavage of Rad9 during apoptosis. *Oncogene*, **22**, 6340–6346.
30. Coleman,P.F., Suttle,D.P. and Stark,G.R. (1977) Purification from hamster cells of the multifunctional protein that initiates *de novo* synthesis of pyrimidine nucleotides. *J. Biol. Chem.*, **252**, 6379–6385.
31. Carrey,E.A., Campbell,D.G. and Hardie,D.G. (1985) Phosphorylation and activation of hamster carbamyl phosphate synthetase II by cAMP-dependent protein kinase. A novel mechanism for regulation of pyrimidine nucleotide biosynthesis. *EMBO J.*, **4**, 3735–3742.
32. Qiu,Y. and Davidson,J.N. (2000) Substitutions in the aspartate transcarbamoylase domain of hamster CAD disrupt oligomeric structure. *Proc. Natl Acad. Sci. USA*, **97**, 97–102.