Substrate Specificity of RdgB Protein, a Deoxyribonucleoside Triphosphate Pyrophosphohydrolase*

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Nicholas E. Burgis and Richard P. Cunningham

From the Department of Biological Sciences, University at Albany, State University of New York, Albany, New York 12222

We have previously reported the identification of a DNA repair system in Escherichia coli for the prevention of the stable incorporation of noncanonical purine dNTPs into DNA. We hypothesized that the RdgB protein is active on 2′-deoxy-N-6-hydroxylaminopurine triphosphate (dHAPTP) as well as deoxyinosine triphosphate. Here we show that RdgB protein and RdgB homologs from Saccharomyces cerevisiae, mouse, and human all possess deoxyribonucleoside triphosphate pyrophosphohydrolase activity and that all four RdgB homologs have high specificity for dHAPTP and deoxyinosine triphosphate compared with the four canonical dNTPs and several other noncanonical (d)NTPs. Kinetic analysis reveals that the major source of the substrate specificity lies in changes in $K_m$ for the various substrates. The expression of these enzymes in E. coli complements defects that are caused by the incorporation of HAP and an endogenous noncanonical purine into DNA. Our data support a preemptive role for the RdgB homologs in excluding endogenous and exogenous modified purine dNTPs from incorporation into DNA.

Our laboratory has shown that an Escherichia coli recA200(Ts) rdgB double mutant is inviable at the nonpermissive temperature and suggested that in the absence of RdgB a lesion develops in DNA that requires repair by a RecA-mediated event (1). Overexpression of the purA gene suppresses the temperature-sensitive phenotype of rdgB recA200(Ts) mutants (2). The purA gene encodes adenylsuccinate synthase, which catalyzes the first step in the conversion of IMP to AMP (3). This result suggests that RdgB may play a role in modulating nucleotide pools. The yeast homolog of RdgB has been named HAM1. Pavlov and co-workers (4, 5) have reported that HAM1 mutants of Saccharomyces cerevisiae display hypersensitivity to N-6-hydroxylaminopurine (HAP) and a hypermutable phenotype upon HAP exposure. They suggested the possibility that HAM1 may be a deoxyribonucleoside triphosphate pyrophosphohydrolase and that it may play a role in detoxifying 2′-deoxy-HAP triphosphate. E. coli cells that are deficient in molybdopterin biosynthesis (moa strains) display a hypersensitive and mutagenic phenotype upon exposure to HAP (6). We have recently reported that inactivation of the rdgB gene in a moa background resulted in increased HAP sensitivity, an increase in the level of mutagenesis, and increased recombination and SOS induction upon HAP exposure (7). Our results support a model for the exclusion of HAP residues from DNA that includes a molybdoenzyme and RdgB protein and suggest that the repair system excludes an unknown endogenous noncanonical purine from DNA.

Recent work in our laboratory has shown that endonuclease V is an important component of a repair pathway for noncanonical purines that have been incorporated into DNA (7). We have demonstrated that inactivation of the nfi gene in E. coli results in a reversal of HAP sensitivity, as well as the hyper-recombination and SOS-induced phenotypes of moa and moa rdgB strains exposed to HAP. Moreover, transduction of an nfi mutation into a moa or moa rdgB genetic background results in a further elevated level of mutagenesis. These results led us to propose that endonuclease V plays a primary role in repairing HAP lesions in DNA. We further hypothesized that the lethal lesion may be a double-strand break created by the action of a replicative polymerase traversing endonuclease V-nicked HAP-containing DNA (7). A similar role for RdgB protein and endonuclease V has been proposed by Bradshaw and Kuzminov (8) in the exclusion of deoxyinosine triphosphate (dITP) from DNA.

The crystal structure of the Methanococcus jannaschii homolog of RdgB (Mj0226) has been solved using a structure-function based experimental design (9). This study showed that the protein exists in solution as a homodimer and has an active site cleft that is ideal for nucleotide binding and hydrolysis. Biochemical analysis showed that the protein displays deoxyribonucleoside triphosphate pyrophosphohydrolase activity and has a preference for noncanonical purine nucleoside triphosphates, with little difference in activity between deoxy- and ribonucleotides (9, 10). It was determined that xanthine triphosphate (XTP) and dITP are, respectively, about 180- and 140-fold better substrates for Mj0226 than the best canonical nucleoside triphosphates, dGTP and GTP. The authors suggested that Mj0226 might play a primary role in cleansing purine deoxyribonucleoside triphosphate pools. Interestingly, it was determined that Mj0226 has extremely low deoxyribonucleoside triphosphate pyrophosphohydrolase activity with dHAPTP (10). This result is in stark contrast to the genetic data reported by Pavlov and co-workers (4) and our laboratory (7)
supporting a role for RdgB in excluding dHAPTP from replication precursor pools.

Biochemical analysis of the human homolog of RdgB, ITPase, has shown that the human homolog possesses biochemical properties similar to MjO226 (11). This study focused on the role of ITPase in preventing accumulation of dITP in human cells. It was shown that ITPase has a 100-fold preference for dITP as a substrate compared with the best canonical purine, GTP or dGTP. Again, it was shown that there was little difference between the deoxy and ribose forms. This study also demonstrated that ITPase is substrate inhibited at high substrate concentrations, as previously documented (12), and forms a 45-kDa homodimer in solution.

Biochemical analysis of the E. coli homolog, EcO197 (RdgB), has shown that it has activity similar to that of the M. jannaschii and human homologs (13). It too has a preference for noncanonical purine nucleoside triphosphates and shows the highest activity with XTP as a substrate, whereas dITP is the next best substrate. EcO197 was determined to have a 100-fold difference in substrate specificity for dITP and dGTP. dGTP and GTP were demonstrated to be the best canonical substrates, and EcO197 was shown to possess pyrophosphate-releasing activity with GTP as a substrate. Effects of substrate inhibition at high substrate concentrations were not identified in this report (13). For simplicity, we will refer to the E. coli homolog as RdgB, the original designation.

Because of the discrepancies between the biochemical data (10) and the genetic data (4, 7) with regards to dHAPTP and the lack of a comparative analysis of the RdgB homologs, we chose to investigate the biochemical properties of the E. coli, yeast, murine, and human homologs. Here we demonstrate that all four of the homologs have similar biochemical properties. All four homologs have a preference for noncanonical purines, have deoxyribonucleoside triphosphate pyrophosphohydrolase activity with dHAPTP as a substrate, and display substrate inhibition at high dITP concentrations. Kinetic analysis revealed that the major source of differences in specificity between canonical and noncanonical substrates was substrate binding. Furthermore, we provide biological evidence of a role for these proteins in the cleansing of purine pools. Overexpression of any of the four RdgB homologs in a recA200(Ts) rdgB double mutant reverses the temperature-sensitive phenotype. We also demonstrate that all four RdgB homologs complement E. coli moa rdgB double mutants with respect to HAP sensitivity. The data we present strongly suggest that the RdgB homologs have a primary role in excluding noncanonical purines from deoxyribonucleoside triphosphate pools, including dHAPTP.

**EXPERIMENTAL PROCEDURES**

**E. coli Growth Conditions**—The medium used for growth was TY (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl/liter at pH 7.2). Antibiotics were used at the following concentrations: tetracycline hydrochloride, 20 μg/ml; kanamycin sulfate, 34 μg/ml; chloramphenicol, 20 μg/ml; ampicillin, 50 μg/ml. Cell growth was routinely monitored in a Genesys 1Vis spectrophotometer (Thermo Spectronic, Rochester, NY) at 600 nm.

**RdgB Substrate Specificity**

**E. coli Strain Construction—P1-mediated transductions were performed as described previously (7). Strains containing a deletion of the purA gene (∆purA) were constructed using a PCR-based technology developed by Link et al. (14) in the KL16 wild type E. coli strain. Deletions of the rdgB gene (∆rdgB) were constructed by the method of Datsenko and Wanner (15). rdgB::FLP(cat-1)FLP alleles were initially constructed in BW25113 cells bearing an arabinose-inducible λ Red helper plasmid. The resulting rdgB insertional mutant was moved into the BL21(DE3) (Novagen, San Diego, CA) expression strain by P1 transduction with selection for chloramphenicol resistance. The chloramphenicol resistance gene was flanked by FLP recognition target sequences, allowing for the rdgB insertional mutants to be converted to deletion mutants with the aid of a helper plasmid bearing a temperature-sensitive replicon and a thermally inducible promoter driving FLP recombinase expression (15). An antibiotic-sensitive clone was isolated and designated NEB195. All of the deletion strains were verified by PCR analysis (14, 15). The recA200 allele was introduced into RPC133 (1) by cotransduction with the linked slrD300::Tn10 allele (16) at 30 °C and selection for tetracycline resistance to generate a rdgB recA200(Ts) strain. moa strains were constructed by transducing the moa-250::Tn10(Tc') allele into W3110 and selecting for tetracycline resistance. All moa mutants were confirmed by testing for chlorate resistance (17). moa rdgB strains were constructed first by transducing the rdgB3::Kan (1) allele into W3110 and selecting for kanamycin resistance followed by introduction of the moa-250::Tn10(Tc') (7) allele by P1 transduction. The moa purA rdgB nfi strain was constructed by moving the following alleles into the ∆purA strain: nfi-1::Cat (18), rdgB3::Kan, and moa-250::Tn10(Tc'). nfi mutant alleles were selected on the basis of chloramphenicol resistance.

**Measurement of HAP Cytotoxicity**—Fresh overnight cultures of strains to be assayed were serially diluted and then plated on TY agar containing various concentrations of HAP. Surviving colonies were scored the next day. All of the strains tested in this assay were derivatives of E. coli W3110.

**Overexpression and Purification of His6-tagged RdgB Homologs**—The coding region of rdgB was cloned from E. coli AB1157 cells into a pET28a vector (Novagen) with NdeI and BamHI restriction sites engineered into the 5′-end of the rdgB coding region. The resulting kb::Tn10(Tc') allele was introduced into BL21(DE3) (Novagen, San Diego, CA) expression strain by P1-mediated transduction with selection for chloramphenicol resistance. The resulting strain was transformed with pET28a from BY4741 cells in a similar manner. The mouse rdgB coding sequence was amplified and cloned by reverse transcription-PCR. Briefly, mRNA was extracted from NIH 3T3 cells with the Oligotex Direct mRNA extraction kit (Qiagen). First strand cDNA was synthesized with Omniscript reverse transcriptase (Qiagen) and NIH 3T3 mRNA template in a reaction primed with oligo(dT). Subsequent PCRs were performed with this cDNA template and the rdgB-specific primer pair rdgB5 (5′-CAG TAA GCT TCT TGC TTG CAA TGG CTG CGT CTT TG) and rdgB3 (5′-ACT CGA ATT CCT ACC CAT GCC GCT AGT GGT CAC CA). The amplified rdgB coding sequence was gel-purified, digested with and HindIII, and cloned into EcoRI/HindIII-digested pcDNA3.1Zeo+. The human rdgB coding sequence was also...
amplified and cloned by reverse transcription-PCR. Briefly, mRNA was extracted from RIG cells with the Oligotex Direct mRNA extraction kit (Qiagen). First strand cDNA was synthesized with Omniscript reverse transcriptase (Qiagen) and RIG mRNA template in a reaction primed with oligo(dT). Subsequent PCRs were performed with this cDNA template and the human rdgB-specific primer pair HrdgB5 (5’-CAG TAA GCT TGA TCA CCA TGG CCG CCT CAT T) and HrdgB3 (5’-ACT CGA ATT CAG CTG CAG AAG TCA AGC TGC CAA ACT). The amplified human rdgB coding sequence was gel-purified, digested with EcoRI and HindIII, and cloned into EcoRI/HindIII-digested pcDNA3.1Zeo. The mouse and human rdgB genes were then cloned into pET28a using a PCR-based cloning strategy as described above. The sequences of the biotic to an E. coli, yeast, mouse, and human rdgB genes in pET28a were determined by DNA sequencing and corresponded to those of the wild type genes. The pET28a-rdgB plasmid was transformed into E. coli BL21(DE3) cells and cultured at 37 °C in the presence of antibiotic to an A600 equal to about 0.6, and recombinant RdgB was overexpressed by isopropyl-β-thiogalactoside induction (Sigma). After 4 h of induction at 37 °C, the cells were harvested by centrifugation, and pelleted cells were stored at −80 °C.

Recombinant yeast, mouse, and human homologs were expressed in a similar manner in NEB195 cells (BL21 (DE3) ΔrdgB). Mammalian rdgB encoding plasmids were cotransformed with pRARE (Novagen). Analysis of cell lysate on an SDS-PAGE gel showed major bands with apparent molecular masses consistent with the predicted molecular masses of 23.9 kDa, 23.7 kDa, and 23.2 kDa for the His6-tagged recombinant yeast, mouse, and human proteins, respectively. Pelleted cells were suspended in 10 mM imidazole, 20 mM phosphate, 0.5 M NaCl buffer at pH 7.4 (Buffer A) and lysed by sonication in five 1-min intervals using a Vibra-Cell sonicator (Sonics & Materials, Inc., Newtown, CT) set at 40% amplitude. The cell lysates were stirred at 4 °C for 1 h, and cell debris was pelleted by centrifugation. His-tagged RdgB homologs were purified from the soluble fraction of these cells on a Ni2+-containing HiTrap® affinity column from GE Healthcare. Supernatant was loaded onto the Ni2+-charged column at a rate of 3 ml/min and washed with ~10 ml of Buffer A. Recombinant protein was eluted in 1-ml fractions with Buffer A containing 500 mM imidazole. Eluted protein was analyzed by SDS gel electrophoresis (19), and a major band displaying the expected molecular weight was observed for each homolog. Fractions showing the highest degree of purity were selected for dialysis. RdgB and HAM1 proteins were dialyzed (1:500 (v/v)) against 10 mM MgCl2, 25 mM CHES, pH 9.5 (Buffer B) overnight. Mammalian homologs were dialyzed against the same buffer containing 1 mM DTT in a similar manner. Yeast, mouse, and human homologs were further purified by size exclusion chromatography using a Superdex 75 gel filtration column (1.6 × 60 cm). The elutions were performed with Buffer B containing 100 mM NaCl. The eluants were analyzed by SDS gel electrophoresis and fractions displaying the highest degree of purity were pooled and dialyzed (1:250 (v/v)) overnight in appropriate buffer. Protein concentration was assayed by the method of Bradford (20) and calibrated with a bovine serum albumin standard. Enzyme preparations were stored in Buffer B and glycerol (1:1 (v/v)) at −20 °C. The final enzyme preparations are displayed on SDS-PAGE in Fig. 1.

**Analysis of Deoxyribonucleoside Triphosphate Pyrophosphohydrolase Activity of RdgB Homologs—**Kinetic analysis was performed with a coupled enzyme assay similar to the protocol described by Bhatnagar et al. (21). Millipore H2O purified with an Easy Pure UV/UF water system from Barnstead-Thermolyne (Dubuque, IA) was used throughout assay and to prepare all solutions. dATP, dCTP, dTTP, dGTP, GTP, and dITP were purchased from Sigma. All other modified nucleoside triphosphates were purchased from TriLink (San Diego, Ca). The reaction mixture contains, in 0.3 ml: 25 mM CHES, pH 9.5, 10 mM MgCl2, 20–2500 μM dNTP, 0.5 unit of yeast pyrophosphatase, and 0.05–5 pmol of enzyme. 50 pmol of recombinant Eco RdgB was used for the substrate specificity reactions. The reactions were preincubated at 37 °C for 10 min, the enzyme was added, and the reaction was allowed to proceed at 37 °C for 10 or sometimes 20 min with dGTP. The reactions were stopped by the addition of 50 μl of 1 N perchloric acid and 50 μl of an aqueous suspension of Norit A (20% packed volume). The suspension was mixed and centrifuged. An aliquot of the supernatant was removed, spin-filtered (Spin-X high pressure liquid chromatography; Corning Inc., Corning, NY) and used for the determination of inorganic orthophosphate by the method of Ames and Dubin (22) with a Genesys 10vis spectrophotometer. Kinetic parameters were determined using the Marquardt-Levenberg algorithm as calculated by the Enzyme Kinetics module of Sigma Plot software.

**NMR Spectroscopy—**We used NMR spectroscopy to characterize dHAPTP dissolved in water. The data were collected on a 400-MHz Bruker NMR spectrometer. Water suppression during the NMR experiment was accomplished using the water gate pulse sequence supplemented with excitation sculpting (23).
**RESULTS**

**RdgB Homologs Complement the Extreme HAP-sensitive Phenotype of moa rdgB Double Mutants**—Cytotoxicity tests were performed to demonstrate that yeast, mouse, and human RdgB homologs complement *E. coli* rdgB strains with respect to a rescue of HAP sensitivity. Rescue of the extreme HAP-sensitive phenotype by RdgB homologs was demonstrated by constructing plasmids with the *E. coli*, yeast, mouse, and human homologs of RdgB cloned into a pBR322-based plasmid so that expression was driven by the *E. coli* endonuclease IV promoter. An empty vector plasmid was also constructed to serve as a control. These plasmids were transformed into *E. coli* strains bearing either *moa* or *moa rdgB* mutations. Fig. 2 shows that *E. coli moa* strains bearing the empty vector plasmid are sensitive to HAP and display ~10% survival when plated on medium containing 50 μg/ml HAP. *moa rdgB* strains bearing the empty vector plasmid are extremely sensitive to killing by HAP and display less than 1% survival when exposed to 5 μg/ml HAP. Fig. 2 shows that cells bearing plasmids expressing an RdgB homolog reverse the extreme HAP-sensitive phenotype of *moa rdgB* double mutants. The rescue is well past the levels of sensitivity displayed by the *moa* mutation alone for overexpression of *E. coli*, yeast, or mouse RdgB protein. With the exception of the human homolog, overexpression of an RdgB homolog results in greater than 80% survival at a HAP concentration of 50 μg/ml. Cells overexpressing the human RdgB complement the *rdgB* genomic mutation less efficiently and displayed a HAP sensitivity phenotype that is equivalent to a *moa* mutation alone. Nonetheless the data presented in Fig. 2 does indeed show that human RdgB can complement the *rdgB* genomic mutation. The human RdgB homolog shows enzymatic activity equivalent to the mouse RdgB homolog (see below), indicating that the reduced level of complementation is most probably caused by a lower level of enzyme expression in the complementation assay. These data support the idea that dHAPTP is indeed a substrate for RdgB and that dHAPTP arises in cells that are deficient in molybdopterin biosynthesis as we have suggested previously (7).

**RdgB Homologs Complement Temperature-sensitive Phenotype of rdgB recA200 Mutants**—Rescue of the *recA* *rdgB* synthetic lethal phenotype by the RdgB homologs was demonstrated by employing the same plasmid constructs that were used in the HAP sensitivity complementation assay. For the temperature sensitivity assay the plasmids were transformed into an *E. coli* strain with an *rdgB recA200* (*Ts*) genetic background. Transformants were streaked on antibiotic containing plates warmed to 30 and 42 °C and incubated overnight at those respective temperatures. Fig. 3 shows that cells bearing plasmids expressing an RdgB homolog are viable at the nonpermissive temperature, whereas cells bearing the empty vector plasmid are not. The fact that these homologs complement the synthetic lethal phenotype of an *rdgB recA200* (*Ts*) strain at 42 °C suggests that the endogenous dNTP(s), which *E. coli* RdgB is preventing from entering replication precursor pools, is a common substrate(s) for all four homologs.

**Determination of Deoxyribonucleoside Triphosphate Pyrophosphohydrolase Assay Conditions**—Purified recombinant bacterial, yeast, mouse, and human proteins were prepared as described under “Experimental Procedures.” As experimental conditions were being defined, we found that assay sensitivity and substrate inhibition limited substrate concentrations for the determination of kinetic parameters to the range of 20–250
Substrate Specificity of E. coli RdgB—Biochemical analysis of the RdgB homologs was performed using a coupled reaction with S. cerevisiae inorganic pyrophosphatase and a colorimetric assay for quantification of inorganic phosphate (22). No pyrophosphate production was detected by the colorimetric assay in the absence of inorganic pyrophosphatase (data not shown). The activity of 50 pmol Eco RdgB with several nucleoside triphosphates at 37 °C was determined. Fig. 4 shows that at 200 μM substrate the difference in activity between dGTP and GTP is statistically insignificant. RdgB has low levels of activity with the other three canonical dNTPs, with dTTP being second best and dATP showing 20-fold less activity than dGTP. Low activity is observed with N6-methyladenine deoxyribonucleoside triphosphate and O6-methylguanine ribonucleoside triphosphate, very low activity is observed with 2,6-diaminopurine deoxyribonucleoside triphosphate, and no activity is observed with 2-aminopurine deoxyribonucleoside triphosphate relative to the activity seen with (d)GTP (Fig. 4). dITP and dHAPTP were assayed under these experimental conditions; however, substrate depletion was observed (data not shown). Because of the robust activity with dITP and dHAPTP, these substrates could not be compared in this manner. Instead, kinetic analysis was performed with dITP, dHAPTP, and dGTP using much lower enzyme concentrations (see below). Our substrate specificity data are similar to results from other biochemical analyses of RdgB homologs (9, 12); however, the activity observed with dHAPTP is in stark contrast to the results of Chung and co-workers (9). To confirm that the dHAPTP we were using was in fact dHAPTP and not dITP or XTP somehow substituted for dHAPTP by mistake, we subjected a sample of dHAPTP to NMR analysis. We were able to assign the 2-H and 8-H protons at 8.2437 ppm and found a broad peak at 7.975 indicative of an OH group on an exocyclic nitrogen. These assignments are in agreement with those we expect from dHAPTP and eliminate the possibility that our preparation of dHAPTP was dITP or XTP, the two other substrates that would show such high reactivity with RdgB protein.

Kinetic Analysis of RdgB Homologs—Table 1 shows results of our kinetic analysis with the four RdgB homologs using dHAPTP, dITP, and dGTP as substrates. With these three substrates all four RdgB homologs obeyed Michaelis-Menten kinetics over the substrate concentrations specified above. Lineweaver-Burk plots of murine RdgB data with dITP, dHAPTP, and dGTP as substrates are shown in Fig. 5. Substrate inhibition was observed at dITP concentrations greater than 250 μM for all four homologs. Fig. 6 shows that classical substrate inhibition occurs with dITP as a substrate for Mmu RdgB at high substrate concentrations. Because of expense, dHAPTP was not assayed under these conditions. Substrate inhibition was observed with dGTP as a substrate only for Hsa RdgB at concentrations greater than 1.6 mM (data not shown). Substrate inhibition and inhibition by contaminating (deoxy)nucleoside diphosphates has been documented previously for the human homolog (11, 24). How-

![FIGURE 4. Substrate specificity of E. coli RdgB. 50 pmol of purified recombinant Eco RdgB was incubated with 200 μM substrate at 37 °C for 10 min in 25 mM CHES, pH 9.5, 10 mM MgCl2. The hydrolysis of substrate was assayed using the colorimetric procedure described under “Experimental Procedures.” The results are depicted as the percentage of activity relative to activity with GTP. The calculations were based on amount of inorganic pyrophosphate produced. Each result is the mean ± S.D. from three independent experiments.](image)
ever, spontaneous hydrolysis of the dITP stock solution was
determined to be negligible by thin layer chromatography
(data not shown). Therefore, it appears that the substrate
inhibition observed for the RdgB homologs is genuine. Data
points that did not appear to reflect substrate inhibition
were chosen to calculate kinetic parameters by a standard
nonlinear least squares estimation technique. The data in
Table 1 show that dHAPTP is a somewhat better substrate
than dITP for Eco RdgB and that these two substrates are
respectively hydrolyzed \(182\) and \(137\) times more efficiently
than dGTP. In contrast, the \(S.\) cerevisiae homolog hydrolyzes
dITP almost three times more efficiently than dHAPTP.
Nonetheless, both dITP and dHAPTP are hydrolyzed much
more efficiently than dGTP and respectively show specificity
constants \(92\) - and \(32\)-fold greater than dGTP. dITP is a
slightly better substrate than dHAPTP for both mammalian
homologs. \(Mmu\) RdgB respectively hydrolyzes dITP and
dHAPTP \(181\)- and \(161\)-fold better than dGTP. Table 1 also
shows that the human homolog is roughly \(30\)% less efficient
at hydrolyzing noncanonical purine deoxyribonucleoside
tliphosphates than the murine enzyme. Nonetheless, the
specificity constants for \(Hsa\) RdgB activity with dITP and
dHAPTP are respectively about \(200\) and \(172\) times greater
than that for dGTP. The activity of the bacterial enzyme is
roughly \(5\)–\(10\)-fold less than that of its eukaryotic counter-
parts for all three substrates (\(Sce\) RdgB and dHAPTP is the
exception). This difference is most likely not due to the
omission of DTT from the nonmammalian reaction mix in
that the yeast enzyme shows activity similar to the mamma-
lian enzymes for dITP and dGTP. Table 1 shows that the
greatest contribution to the differences in substrate specific-
ity between canonical and noncanonical purine deoxyribo-
nucleoside triphosphates results from \(17\)–\(58\)-fold changes in
\(K_m\), whereas the rate of catalysis for the three substrates
assayed remains relatively unchanged for the yeast enzyme,
and differences of less than 10-fold are observed for the other homologs.

**DISCUSSION**

The results presented here support the models proposed by us (7) and others (4) for the exclusion of noncanonical purines from nucleotide precursor pools by RdgB and the RdgB homologs. Our complementation data suggest that all four RdgB homologs protect cells from incorporation of noncanonical purines into DNA. The observation that expression of the yeast, mouse, and human RdgB homologs suppresses the synthetic lethal phenotype of *rdgB recA* *E. coli* strains suggests that all four organisms share a common threat from an endogenous noncanonical dNTP. Furthermore, expression of the four homologs results in protection of *E. coli moa rdgB* cells from the toxicity of HAP when added to growth medium. Therefore, it appears that the RdgB homologs are also capable of protecting cells from the threat of exogenous noncanonical purines.

ITP and dITP are thought to arise endogenously and may enter into RNA and DNA precursor pools during periods of intense purine catabolism or under conditions that result in high oxidative stress (3, 25, 26). The fact that dITP is hydrolyzed by the human RdgB as much as 200-fold better than dGTP suggests that RdgB may have evolved to defend cells from this particular threat. Interestingly, we see that all four RdgB homologs are also efficient at hydrolyzing dHAPTP to the monophosphate form. HAP has been documented to arise in hepatic microsomes of rats by the activity of cytochrome P-450 isoenzymes (27); however, it has not been documented to constitute a significant portion of purine pools. Therefore, it seems more likely that the main function for RdgB is to exclude ITP and XTP from RNA precursor pools and dITP and dXTP from DNA precursor pools.

Our data support the idea that the RdgB homologs preferentially hydrolyze dITP and dHAPTP to the monophosphate form, releasing pyrophosphate and acting to exclude these dNTPs from replication precursor pools. Substrate specificity data show that RdgB has little activity against several other purine nucleoside triphosphates that are modified at the 6- and 2-positions. RdgB is remarkably well evolved for distinguishing purine nucleoside triphosphates that are modified at the 6- and 2-positions. RdgB is remarkably well evolved for distinguishing purine nucleoside triphosphates that are modified at the 6- and 2-positions. RdgB is remarkably well evolved for distinguishing purine nucleoside triphosphates that are modified at the 6- and 2-positions. RdgB is remarkably well evolved for distinguishing purine nucleoside triphosphates that are modified at the 6- and 2-positions. 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Several lines of evidence indirectly implicate RdgB in playing an active role in excluding noncanonical purines from DNA. For example, *E. coli rdgB* mutants display increased spontaneous recombination rates and elevated SOS induction, as well as increased HAP sensitivity, mutagenesis, recombination, and SOS induction for *E. coli moa rdgB* mutants upon HAP exposure (1, 7). Our results and the results of others have demonstrated that RdgB does not show a preference for deoxy- or ribonucleotides, and the notion that RdgB may play an active role in excluding noncanonical purine nucleotides from RNA cannot be dismissed. Because multiple transcripts can arise from expression of a single copy of a gene and the myriad of mechanisms to degrade damaged RNA transcripts, detection of a phenotype that arose from tainting of RNA precursor pools by the absence of RdgB would be difficult and would be expected to result in a pleiotropic phenotype. Nonetheless, noncanonical purines may arise in RNA of *rdgB* strains. Identification of noncanonical purine bases in RNA is required to determine the contribution of RdgB in cleansing RNA precursor pools.

The *Km* values of *E. coli* RdgB protein for dHAPTP and dITP, 16 and 22 *µM*, respectively, are in the range of the concentrations of the canonical dNTPs in *E. coli*, 20–200 *µM* (28), whereas the *Km* for dGTP, 772 *µM*, is well above this concentration. It does not necessarily seem logical that the concentration of a noncanonical dNTP would approach the concentrations of normal dNTPs before RdgB protein would effectively hydrolyze the noncanonical dNTP. However, if we consider that the *Vmax/Km* of DNA polymerase I for dATP is 21 M−1 s−1, whereas the *Vmax/Km* for dHAPTP is 4 M−1 s−1 (29), it appears that dHAPTP is not as efficiently incorporated into DNA as dATP. Thus, RdgB protein need only keep the concentration of noncanonical triphosphates low enough to prevent efficient incorporation by DNA polymerases.

Substrate inhibition of all four enzymes occurs at concentrations of dITP above 250 *µM*. Because this concentration would not be expected to be achieved *in vivo*, we do not believe that the phenomenon of substrate inhibition plays a physiological role in the cell.

The data presented in this report complement the biochemical and genetic data that have been previously

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**FIGURE 7. Model for contamination of DNA replication and/or RNA transcription precursor pools by endogenous and exogenous noncanonical purines in *E. coli*.** Noncanonical purines spontaneously arise intracellularly from the purine salvage and interconversion biosynthetic pathways. Biosynthetic enzymes are identified by their gene symbols: *adk*, adenylate kinase; *gmk*, GMP kinase; *gpt*, guanine phosphoribosyltransferases; *gsk*, guanosine kinase; *guaA*, GMP synthetase; *hpt*, hypoxanthine phosphoribosyltransferases; *moa*, molybdeopterin cofactor biosynthesis; *ndk*, nucleoside diphosphokinase; *ndrAB*, nucleotide reductase; *purA*, adenosylsuccinate synthetase; *purB*, adenosylsuccinate lyase; *purP*, adenine transport system; *P-450, cytochrome P-450 isoenzymes*. Hypothesized enzymatic reactions are in **bold type**. Dashed lines represent nucleotide shutting into replication precursor pools. The **black arrow** represents replication and/or transcription. This figure was partially adapted from Zalkin and Nygaard (3).
reported concerning this repair system. Our previous genetic studies in *E. coli* implicated RdgB in removing dHAPTP from replication precursor pools thereby, preventing mutagenesis and toxicity by HAP (7). Similarly, our data suggested that RdgB prevents the incorporation of an unknown noncanonical purine into DNA, which we speculated could be hypoxanthine (7). Bradshaw and Kuzminov (8) have independently come to a similar conclusion. They reported that DNA isolated from rdgB mutants contains endonuclease V-recognizable modifications and that chromosomal fragmentation was observed in rdgB recBC mutants but was suppressed in rdgB recBC nfi mutants. The substrate specificity of endonuclease V includes hypoxanthine and xanthine containing DNA; therefore, it was suggested that RdgB intercepts dITP and dXTP from incorporation into DNA. Several other reports have demonstrated RdgB homologs to have optimal deoxyribonucleoside triphosphate pyrophosphohydrolase activity with (d)ITP and XTP as substrates (8–10, 12); however, this is the first report to demonstrate dHAPTP as a substrate for any RdgB homolog, as has been postulated for some time (4). Taken together, our results support the idea that a functional system to prevent the stable incorporation of endogenous and exogenous noncanonical purines into DNA may be important for the survival of *E. coli* and other organisms (Fig. 7).

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