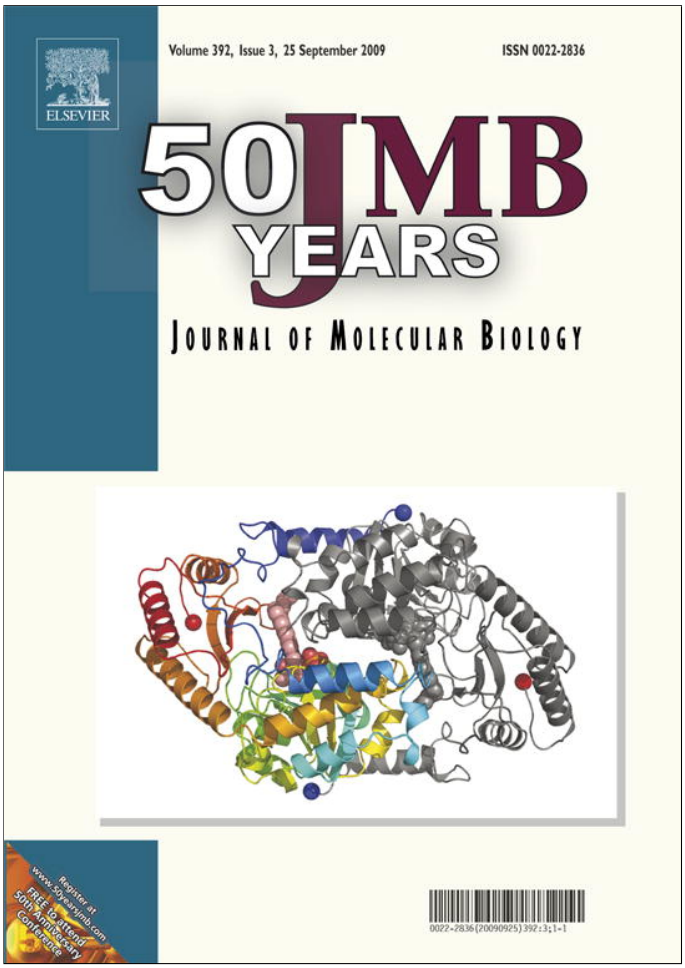

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Functional Study of the P32T ITPA Variant Associated with Drug Sensitivity in Humans

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Sanitization of the cellular nucleotide pools from mutagenic base analogues is necessary for the accuracy of transcription and replication of genetic material and plays a substantial role in cancer prevention. The undesirable mutagenic, recombinogenic, and toxic incorporation of purine base analogues [i.e., ITP, dITP, XTP, dXTP, or 6-hydroxylaminopurine (HAP) deoxynucleoside triphosphate] into nucleic acids is prevented by inosine triphosphate pyrophosphatase (ITPA). The *ITPA* gene is a highly conserved, moderately expressed gene. Defects in *ITPA* orthologs in model organisms cause severe sensitivity to HAP and chromosome fragmentation. A human polymorphic allele, 94C→A, encodes for the enzyme with a P32T amino acid change and leads to accumulation of non-hydrolyzed ITP. ITPase activity is not detected in erythrocytes of these patients. The P32T polymorphism has also been associated with adverse sensitivity to purine base analogue drugs. We have found that the ITPA-P32T mutant is a dimer in solution, as is wild-type ITPA, and has normal ITPA activity *in vitro*, but the melting point of ITPA-P32T is 5 °C lower than that of wild-type. ITPA-P32T is also fully functional *in vivo* in model organisms as determined by a HAP mutagenesis assay and its complementation of a bacterial *ITPA* defect. The amount of ITPA protein detected by Western blot is severely diminished in a human fibroblast cell line with the 94C→A change. We propose that the P32T mutation exerts its effect in certain human tissues by cumulative effects of destabilization of transcripts, protein stability, and availability.

Keywords: nucleotide pools; ITPA; base analogs; drug sensitivity

Introduction

Nucleotides of hypoxanthine and xanthine have multiple functions in all organisms. Inosine monophosphate (IMP) is synthesized in cells by enzymes of the *de novo* purine biosynthesis pathway, which is universal in all organisms and best studied in yeast and bacteria.[1,2] Hypoxanthine, xanthine, and their derivatives are also generated at different steps of the purine salvage pathway, utilizing exogenous and endogenous purines. IMP serves as a precursor for the synthesis of adenine and guanine nucleotides, required for energy metabolism and biosynthesis of DNA, RNA, cofactors, signal messengers, and hormones. The triphosphate forms of hypoxanthine (dITP) or xanthine (dXTP) are undesirable because they could be incorporated into nucleic acids instead of canonical nucleotides and cause genetic damage.[3,4] One source of intracellular ITP is the

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Abbreviations used: ITPase, generic name of enzymes hydrolyzing ITP; ITPA, human ITP pyrophosphatase; IMP, inosine monophosphate; HAP, 6-hydroxylaminopurine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

activation of IMP by machinery used for other nucleoside monophosphates.[2] Another source of hypoxanthine nucleotides in the cell is spontaneous or induced (e.g., by oxidative stress or inflammation) deamination of adenine nucleotides.[5–7]

As structural analogues of dATP and dGTP, the noncanonical dITP and dXTP nucleotides are incorporated into DNA during DNA replication. Incorporation of dITP from the pools opposite template C is non-mutagenic, similar to the incorporation of dUTP;[8] misincorporation of dXTP impedes the progression of replication.[9] On the other hand, on the DNA level, the deamination of adenine to hypoxanthine or guanine to xanthine results in elevated mutagenesis[10,11] because hypoxanthine pairs as G[12] and xanthine is a DNA replication blocking lesion. Irrespective of the route of appearance in DNA, both inosine and xanthine are recognized in most organisms by a specialized repair system initiated by the orthologs of endonuclease V[13] and elicit DNA repair reactions that lead to DNA fragmentation and genomic instability when the level of analogues is high.[3,14,15]

The efficient system for the control of the concentration of hypoxanthine and xanthine from bacteria to mammalian cells is based on the action of specific triphosphatases. The prominent enzyme sanitizing the DNA precursor pool is inosine triphosphatase (ITPA), which prevents the accumulation of ITP, XTP, dITP, and dXTP in the cell. ITPA catalyzes the hydrolysis of these triphosphates to corresponding nucleoside monophosphates and inorganic pyrophosphate. The genes encoding ITPase are conserved in evolution from bacteria and archaea to humans.[3,16] All investigated proteins of the ITPase family have similar biochemical properties (see Ref. 17 and references therein).

Interestingly, the inactivation of genes encoding ITPA leads to different phenotypes in different species. Phenotypic characteristics of ITPase-defective mutants were described long before they were linked to the gene encoding ITPase. In yeast *Saccharomyces cerevisiae*, ITPase is encoded by the *HAM1* gene. Mutations in the *HAM1* gene were found in the screen for elevated sensitivity of yeast strains to the toxic and mutagenic effects of the base analogue 6-hydroxylaminopurine (HAP).[18] The gene disruption mutation of the *HAM1* gene is viable, does not lead to elevated spontaneous mutagenesis, and has no other phenotypes besides HAP hypersensitivity.[19] In *Escherichia coli*, ITPase is encoded by the *rdgB* gene. The *rdgB* mutants were first identified by their lethal effect on the *recA200*(Ts) background at the nonpermissive temperature.[20] Inactivation of the *rdgB* gene alone does not lead to increased sensitivity to HAP because of an additional powerful HAP-protecting system dependent on the molybdenum cofactor in bacteria.[21,22] The effect of the *rdgB* inactivation on HAP sensitivity is clear in strains defective in the biosynthesis of the molybdenum cofactor.[4] In these strains, *rdgB* mutations lead to increased recombination, DNA fragmentation, and SOS induction.[3,4,15] The link between the phenotypes in microorganisms described previously and ITPase deficiency was established when the crystal structure of the protein encoded by the gene *Mj0226* from a thermostable bacterium *M. jannaschii* homologous to *rdgB* and *HAM1* was determined.[23] Mj0226 was found to be a dimer of identical subunits with some structural features in common with the MutT and MTH1 triphosphate pyrophosphohydrolases, suggesting that ITPA is a member of a new subclass of dNTPases. Biochemical studies confirmed that ITPase from *E. coli*, yeast, mouse, and human hydrolyzes deoxynucleosides and ribonucleosides of hypoxanthine, xanthine, and HAP much more effectively than canonical nucleotides.[24–28]

In humans, ITPase is encoded by the *ITPA* gene located on the short arm of chromosome 20.[25] The *ITPA* gene is orthologous to microbial genes *Mj0226*, *rdgB*, and *HAM1*. Several allelic variants of the *ITPA* gene have been described in different human populations: the coding sequence *ITPA* 94C→A causing a P32T change in the corresponding protein and changes in introns, *ITPA* IVS2+21A→C, *ITPA* IVS2+68T→C and *ITPA* IVS2+68T→G alleles.[29–35] All of these variants are associated with different ranges of decreased ITPase activity. It was shown that the concentration of ITP is dramatically increased to a readily detectable level in erythrocytes of ITPase-deficient patients. No pathological conditions have been found thus far in those individuals. Several reports connect low or zero ITPase activity in red blood cells of 94C→A allele carriers and adverse drug reactions. For example, there is intolerance of therapeutic thiopurines (6-mercaptopurine and azathioprine) that are often used in treatment of inflammatory diseases and acute lymphoblastic leukemia and for prevention of organ rejection after transplantation.[36–40] This association was questioned[41] and prompted further population studies.[42] More recently, a significantly higher probability of severe febrile neutropenia was observed in patients with a variant ITPA allele among patients whose dose of mercaptopurine had been adjusted for the thiopurine *S*-methyltransferase genotype.[40] A new role of ITPase in regulation of levels of ATP for proper muscle contraction was proposed based on recent finding of heart failure in *Itpa* knockout mice.[43] Because of the strong connection between ITPase deficiency and certain medical conditions, it is important to understand the mechanisms of substrate specificity of ITPase and its variants in direct biochemical experiments.

One obvious question is the basis of ITPase activity loss in patients with a 94C→A allele variant of the *ITPA* gene. Recently, a crystal structure of human ITPA has been solved.[17,44] The P32 residue is located in the loop connecting helix α1 and β-strand 2 (Fig. 1a), which is located away from the active site of the ITPase and is not conserved among species.[13,20] The change could affect catalysis by an allosteric mechanism and perhaps disrupt the proper folding of the whole protein. Another hypothesis is that P32T substitution disrupts the proper assembly or cross talk between the two subunits.[17] This idea is

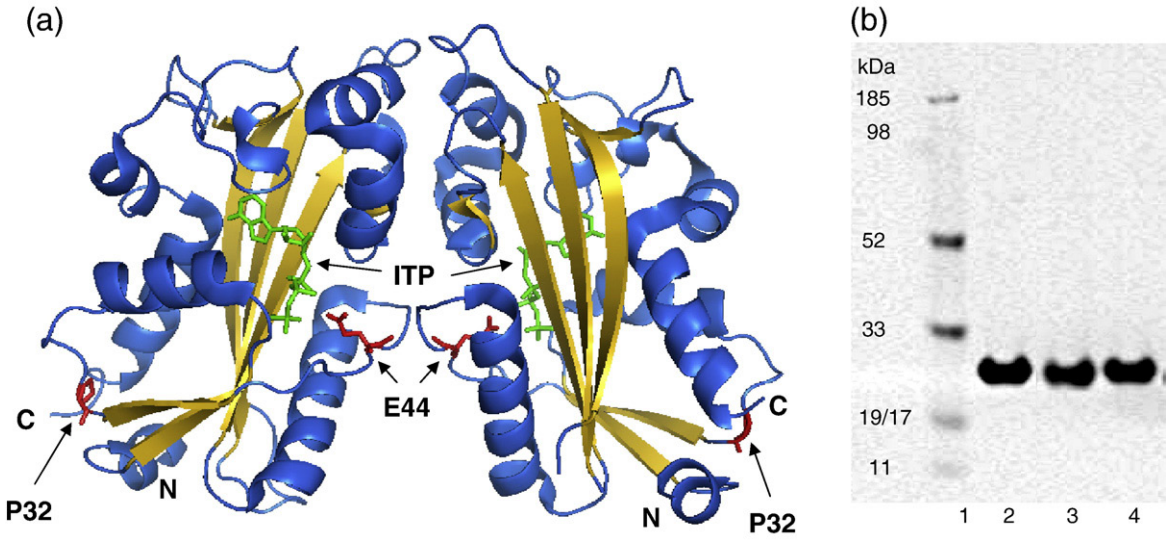

Fig. 1. Human ITPA and its variants. (a) The location of P32T and E44L mutations on the crystal structure of human ITPase. For the dimer structure, P32 and E44 residues are in red and marked with arrows, and ITP is in green. The secondary structure of ITPase is shown with a ribbon diagram colored with β-strands (yellow) and all other elements (blue). The locations of N and C termini are labeled. The dimer interface is located in the center of the figure. (b) Purified proteins separated on SDS-PAGE gel and visualized with Coomassie stain. Lane 1, MultiMark (Invitrogen) molecular weight marker; lane 2, wild-type ITPA; lane 3, ITPA-P32T; lane 4, ITPA-E44L.

consistent with the observation that homozygotes for the 94C→A mutation have no ITPase activity in red blood cells, whereas in heterozygotes, the ITPase is retained at about 22.5% of the wild-type activity.[32] The result is predicted by random combinations of the two subunits of the enzyme, where only homodimers of the wild-type subunits are active. It is also possible that NTP hydrolysis in the two monomers is coupled across the dimer interface. Examination of the wild-type ITPase structure indicates that the loop with the P32T change is not part of the dimer interface (Fig. 1a); thus, the mutation is unlikely to disrupt the interaction between subunits but could exert a dominant-negative effect on the ITPase reaction. The third possible reason for the absence of ITPase activity in 94C→A patients is a defect in mRNA processing.[34] None of the proposed mechanisms have been rigorously tested experimentally.

We have found that purified human ITPA-P32T mutant protein is dimeric in solution and possesses pyrophosphatase activity comparable to that of the wild-type protein. The 94C→A allele compensates for the HAP sensitivity of *ham1* and *rdgB* mutations in *S. cerevisiae* and *E. coli*, similar to the wild-type *ITPA*. We propose that the reason of complete ITPase deficiency in humans with a P32T change is a catastrophic coincidence of several moderate changes of gene expression and protein properties.

Results

ITPase activity studies

To measure ITPase activity of purified proteins (Fig. 1b) on their presumed natural substrates, dITP and ITP, we used the colorimetric assay described in the Materials and Methods section. Because ITPase is known to be inhibited by high concentrations of substrates (from 0.25 to 3.5 mM in different studies),[25,28] we first determined the concentration dependence of the ITPase activity. Under our experimental conditions (see Materials and Methods), we observed substrate inhibition of both wild-type and P32T mutant proteins at a concentration of ITP above 1 mM (Fig. 2). For dITP, ITPA-P32T was more sensitive to the substrate inhibition (ANOVA, $P<0.01$; Fig. 2b). There is no difference between mutant and wild-type enzymes for ITP (Fig. 2a). Protein with the amino acid change E44L, removing the carboxylate that from the crystal structure is expected to abolish Mg^{2+} binding, was inactive (data not shown). The lack of activity of ITPA-E44L, which is unable to perform a catalytic reaction, suggests that our affinity column purification protocol is specific to the His-tagged human enzyme and does not result in co-purification of bacterial ITPase. For kinetic constant measurements, we used 12 concentration points. The resulting values are presented in Table 1. Surprisingly, the P32T protein had almost the same activity as normal ITPase.

Dimerization status of ITPA P32T in solution

We used size-exclusion chromatography–multi-angle light scattering (SEC–MALS) to examine the molecular weight of wild-type and P32T-ITPA in solution. Monomers of wild-type and P32T-ITPA have an approximate molecular mass of 21.73 kDa, thus giving an expected dimer mass of 43.46 kDa. Wild-type and P32T-ITPA were eluted as a single peak with a molecular mass consistent with a dimer (Supplementary Fig. S1a and b, top). SDS-PAGE

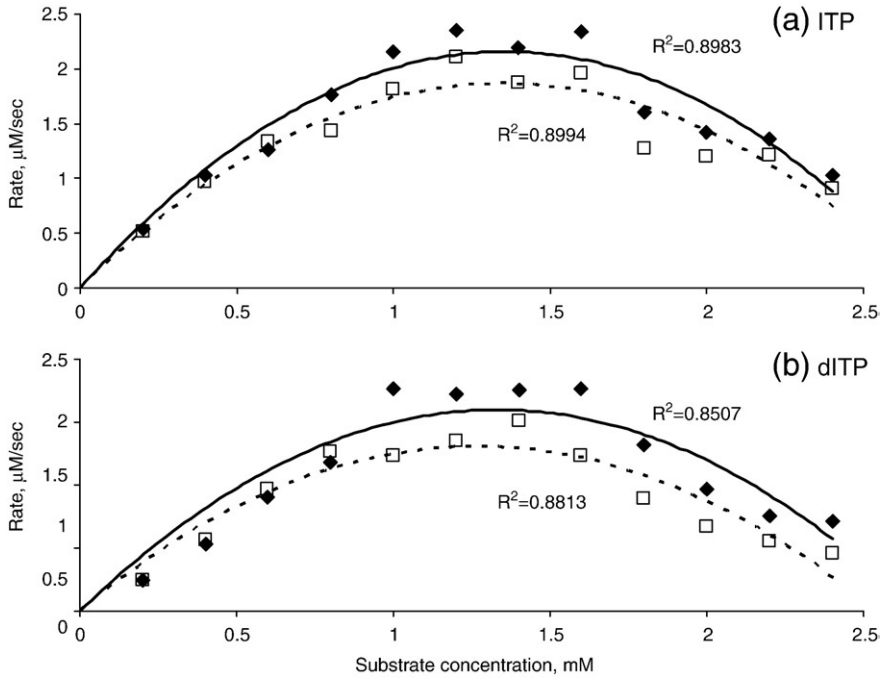

Fig. 2. The dependence of ITPase activity on the concentrations of substrates, ITP and dITP. Activity assay was performed as described in Materials and Methods. High R^2 values close to 1 confirm the validity of approximation. Filled diamonds, wild-type ITPA; open squares, ITPA-P32T.

followed by silver staining confirmed that the peak contained a single species running at a molecular weight consistent with monomeric ITPA (Supplementary Fig. S1a and b, bottom). If the P32T mutation affected the ability of ITPA to dimerize, it would be expected that, especially at low concentration, ITPA-P32T would exist, at least partially, as a monomer. Wild-type ITPA and ITPA-P32T were examined over a 50-fold concentration range and invariably eluted as a single species that had a molecular weight consistent with a dimer (Table 2). Our data with different concentrations of ITPA demonstrate that ITPA-P32T is efficient at dimerization.

Decreased thermostability of ITPA-P32T

We have examined the temperature-induced denaturation profile of ITPA and its P32T variant and found that the P32T variant is less thermostable than the wild-type protein (Fig. 3). Complete denaturation of the wild-type ITPA is achieved at 53–57 °C, depending on concentration (Fig. 3a). P32T-ITPA consistently denatures at about 5 °C lower temperatures over the concentration range tested (Fig. 3b).

Table 1. Kinetic parameters of human wild-type ITPA and ITPA-P32T

Protein	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Wild type	dITP	0.35±0.05	71±10	200±38
	ITP	0.40±0.07	91±23	228±72
P32T	dITP	0.21±0.03[a]	38±7[a]	178±38
	ITP	0.48±0.09	103±24	216±65

The values are averaged from at least three experiments with SE.
[a] This value is statistically different from the other values in the same column as determined by Student's t test ($P<0.05$).

Expression of wild-type ITPA and ITPA-P32T in bacteria and yeast

In biochemical tests *in vitro*, we have shown that the amino acid substitution P32T does not reduce ITPase activity. This result is not expected from human population studies, which show that individuals homozygous for the 94C→A mutation result in null ITPase activity in erythrocytes. To test the hypothesis that mutation leading to P32T amino acid change may reduce ITPase activity *in vivo*, we studied the effect of heterologous expression of the *ITPA* gene in bacteria *E. coli* and yeast *S. cerevisiae*. From previous studies, it is known that the ITPase encoded by the yeast *HAM1* gene and human *ITPA* gene retains their enzymatic activity when expressed in *E. coli*.[28,45] Here we tested the ability of the wild-type and mutant alleles of the human *ITPA* genes encoding for P32T and E44L amino acid changes to protect from HAP-induced killing and

Table 2. Molecular weight in solution of wild-type ITPA and ITPA-P32T as determined by SEC–MALS

Wild type		P32T	
Concentration (mg/mL)	Molecular mass (kDa)	Concentration (mg/mL)	Molecular mass (kDa)
10.08	45.92±0.37	8.74	43.75±0.09
1.01	46.47±0.19	0.87	44.00±0.22
0.20	42.33±0.85	0.17	44.07±0.88

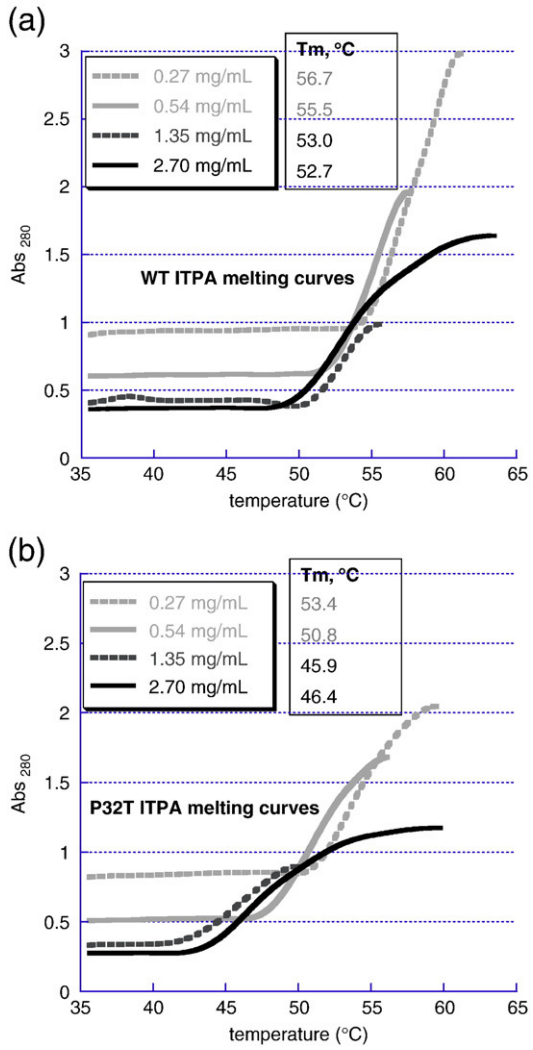

Fig. 3. UV thermal denaturation curves of purified wild-type ITPA and ITPA-P32T. Solutions of wild-type ITPA (a) or ITPA-P32T (b) at the indicated concentrations were heated while monitoring the absorbance at 280 nm. Plots are truncated at the peaks, after which precipitation of the protein led to a decline in absorbance. Melting temperatures, which occur at the inflection point of the plots, were determined by identifying the temperature at the peaks in first-derivative plots.

mutagenesis and compensate for the inactivation of microbial orthologs *rdgB* from *E. coli* and *HAM1* from *S. cerevisiae*.

In bacteria, mutations of the *rdgB* gene are incompatible with the *recA* gene defect.[20] We used the *E. coli* strain EK5 described before (Materials and Methods). This strain has two mutations, *rdgB* allele Δ*yggV62* and *recA200*(Ts), and does not grow at the nonpermissive temperature, 42 °C. We expected the expression of functional ITPase in this strain to complement the *yggV* and rescue the temperature sensitivity. We plated serial dilutions of EK5 transformants by pET15b vector with the wild-type *ITPA* gene or mutant alleles (encoding for P32T and E44L changes) onto minimum media with IPTG for induction of *ITPA* expression. Then we grew them at permissive and nonpermissive temperatures (Fig. 4a). All transformants grew well at 30 °C (left image). The expression of the wild-type *ITPA* gene completely rescues the temperature sensitivity of the EK5 transformants (two top clones on the right image). Mutant allele *ITPA-E44L* encoding for catalytically dead ITPA-E44L does not compensate for the lethal effect of a double mutation combination (two middle rows). Transformants with plasmids with the mutant allele encoding for P32T were indistinguishable from the wild type (two bottom rows). Our data show that human ITPase and its P32T variant, when produced in *E. coli*, are similarly functionally active in the ability to compensate the *rdgB* defect. The result is consistent with the data on the catalytic proficiency of the purified P32T protein. We confirmed this observation in two additional tests in bacteria with the use of HAP. In one test, we have studied the ability of the human *ITPA* gene to suppress HAP-induced killing of *E. coli* Rosetta strain. The Rosetta strain is HAP hypersensitive, likely due to the presence of the *gal* deletion encompassing some part of the *mod* operon, which disturbs molybdenum uptake and leads to a molybdenum cofactor deficiency.[22,46] As is seen in Fig. 4b, wild-type *ITPA* and *ITPA-P32T* mutants of the *ITPA* gene protect the Rosetta strain from HAP. The plasmid with allele encoding for the E44L change within the active site of ITPA, which results in the abrogation of catalytic activity (the results of biochemical study of ITPA), does not affect the hypersensitivity of Rosetta to HAP. In the other test, we studied the effects of *ITPA* expression on HAP-induced mutagenesis in the wild-type bacterial strain EK1. In this test, HAP was spotted onto the center of the plate with a lawn of bacteria (as in Fig. 4b), and after 24 h of incubation, plates were replica-plated onto LB plates with antibiotic rifampicin. HAP is a potent inducer of forward mutations to rifampicin resistance, as judged by the appearance of numerous resistant colonies surrounding the spot of application of the mutagen. The expression of wild-type *ITPA* suppressed the mutagenic effect almost completely (Fig. 4c, top row, compare plates without and with IPTG). The expression of *ITPA-E44L* did not affect HAP-induced mutagenesis at all (middle row), while the expression of *ITPA-P32T* led to a similar level of protection to that with the expression of wild-type *ITPA* (bottom row). Taken together, the data presented in Fig. 4 are consistent with the full functionality of ITPA P32T, both in regard to natural substrates and in regard to HAP toxicity and mutagenesis.

Expression of ITPA wild-type and ITPA-P32T mutant alleles in yeast

We expressed human alleles encoding for wild-type ITPA and ITPA with amino acid substitutions P32T and E44L under a galactose promoter in pESC-URA vector in yeast and have shown that the *ITPA* wild type and the *ITPA-P32T* mutant complement HAP-induced mutability of a yeast *ham1* mutant

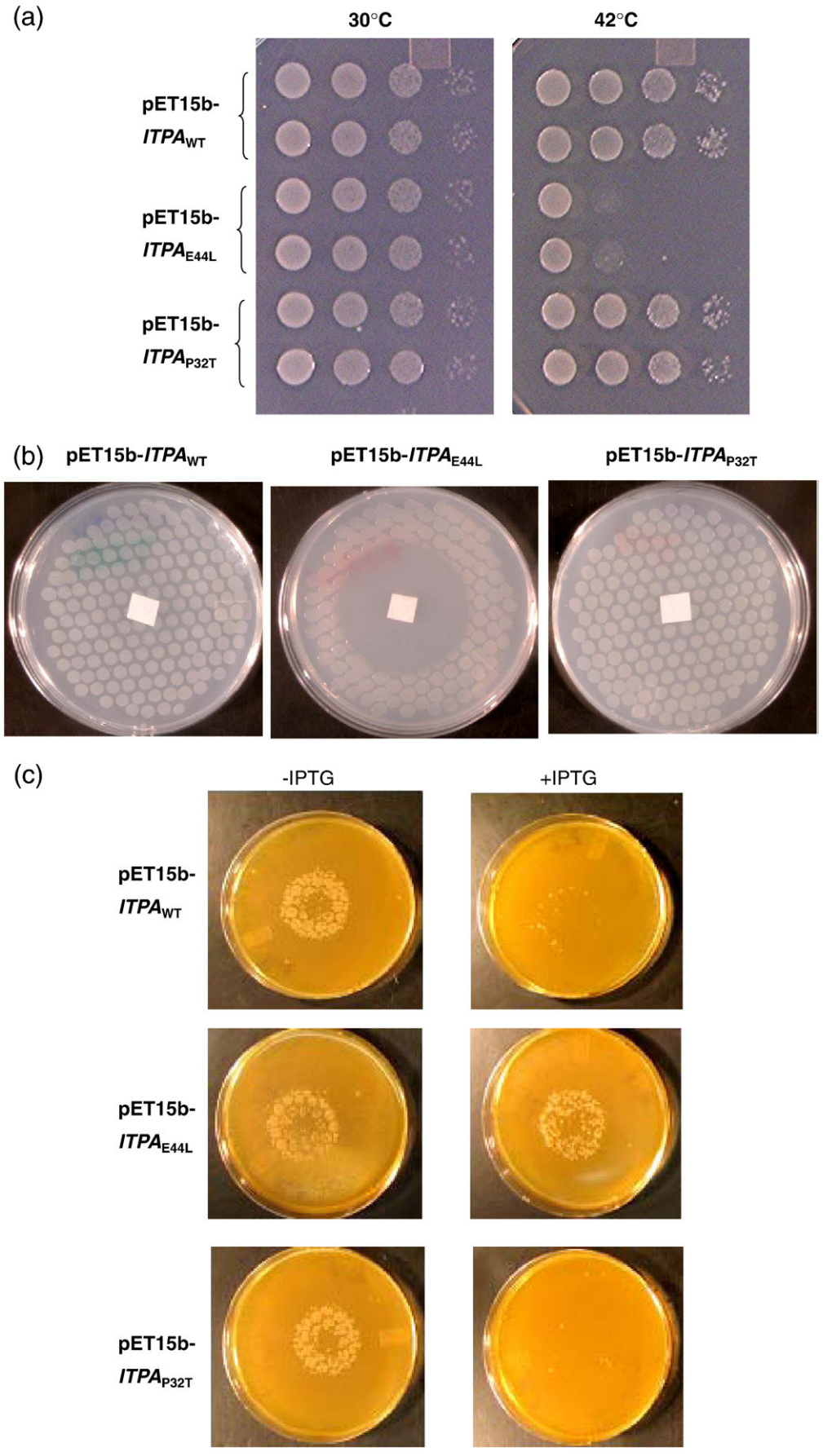

Fig. 4 (*legend on next page*)

(Fig. 5). A yeast strain with the *ham1* mutation and with empty vector or with the vector with the ITPA-E44L allele encoding for inactive ITPase is hypersensitive to the mutagenic effect of HAP. Plasmids with the yeast *HAM1* gene or human *ITPA* or *ITPA-P32T* all protected this strain from HAP-induced mutagenesis to the same level—the dose–response curves are identical.

Low levels of ITPA-P32T in human fibroblasts

We compared the levels of soluble immunoreactive ITPase in normal WI-38 and in P32T fibroblasts (Fig. 6, two repeats are presented). The level of ITPA is almost 10-fold lower in P32T fibroblasts (top, gray arrow), while the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were essentially the same (bottom, gray arrow).

Discussion

In the present study, we evaluated a recently recognized system of protection from base analogues by ITPase. The *ITPA* gene is important to study from both the medical and basic science points of view. Cleansing of potentially mutagenic nucleotide analogues from the precursor dNTP pool is an important prerequisite for high-fidelity DNA replication.[47–49] Inaccurate DNA replication and repair leads to cancer and other diseases.[50–52] Environmental exposures and biochemical reactions during oxidative stress and inflammation damage natural nucleoside triphosphates.[53–57] Such mutagenic contaminants in dNTP pools lead to elevated mutation rates and a risk of cancer and other diseases.[58] ITPA is involved in the regulation of the quality of the pool of purine nucleotides.[4] Disturbance of this enzyme leads to cellular accumulation of noncanonical nucleoside triphosphates such as ITP, XTP, and others, which instigate elevated chromosome fragmentation and mutagenesis. ITPA has pharmacogenetic significance because of adverse drug reactions in patients with decreased ITPA activity, which is associated with a point mutation leading to a P32T amino acid change.[40] Here we examined the possible mechanisms of these effects.

We have unexpectedly found that ITPA-P32T is very similar (but not identical) to wild-type ITPA. This conclusion is based on the kinetic characteristics of the protein, its melting profile, and the ability of corresponding alleles to compensate for the ITPase defects in model organisms.

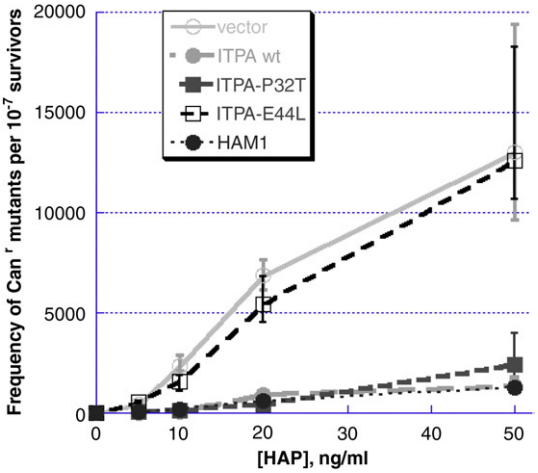

Fig. 5. ITPA-P32T is functional in yeast *Saccharomyces cerevisiae*. Suppression of HAP-induced mutagenesis in *ham1* mutant transformed with plasmid expressing the yeast *HAM1* gene and the human wild-type (WT) *ITPA* gene and its mutant alleles *ITPA-P32T* and *ITPA-E44L*. Transformants with empty vector pESC-URA were used as a control. For frequency determination, we used six independent cultures. The experiment was repeated three times and the data appeared to be homogeneous. Medians of mutant frequencies for each data point were then found. Error bars represent 95% confidence limits.

There are just a few papers describing the kinetic characteristics of human ITPA. There is some variability of estimates. K_m values vary ~10-fold; for example, for dITP, it is 32.5 μM in Ref. 28 and is 310 μM in Ref. 25. The K_m values we observed in our study for both ITP and dITP are very close to those of Lin *et al.*[25] This might relate to slightly different reaction conditions such as buffer used and pH. As for the kinetic parameters of the mutant enzyme, we observed a significant difference between P32T and the wild type only for dITP (Table 1). The enzyme kinetics did not fit the Michaelis–Menten equation because of substrate inhibition. Therefore, we have used for the estimation of kinetic parameters a more adequate special model built for such cases (Materials and Methods). The use of this model allowed us to better describe the differences in substrate inhibition between wild-type and mutant ITPA. It is interesting that although the k_{cat} value for wild-type ITPA is almost twice as high as that for P32T, there is no significant difference between the corresponding k_{cat}/K_m values. This may reflect the stronger sensitivity of the mutant enzyme to the substrate inhibition (Fig. 2). Unlike the other kinetic

Fig. 4. ITPA P32T is functional in bacterium *E. coli*. (a) Sensitivity to high temperature of double mutant Δ*rdgB recA200* (Ts) strain (EK5) transformed with vector expressing the human wild-type (WT) *ITPA* gene and its mutant variants *ITPA-P32T* and *ITPA-E44L*. Five microliters of the serial dilutions of the overnight cultures grown at 30 °C was spotted on minimal medium plates containing 50 μM IPTG. The plates were incubated overnight at 30 °C or at 42 °C. (b) The ability of ITPA-P32T to compensate for the HAP sensitivity of the Rosetta strain. Cells were spotted on minimal medium plates containing 50 μM IPTG using a multi-prong replicator device, and 50 μg of HAP was spotted onto the filter paper at the center of each plate. The plates were incubated overnight at 37 °C. (c) Protection of the wild-type strain EK1 from HAP-induced mutagenesis by human ITPA and its variants. Spot tests were performed as in (b) on minimal medium plates with or without ITPG. The cells were then replica-plated onto LB plates containing rifampicin and incubated overnight at 37 °C.

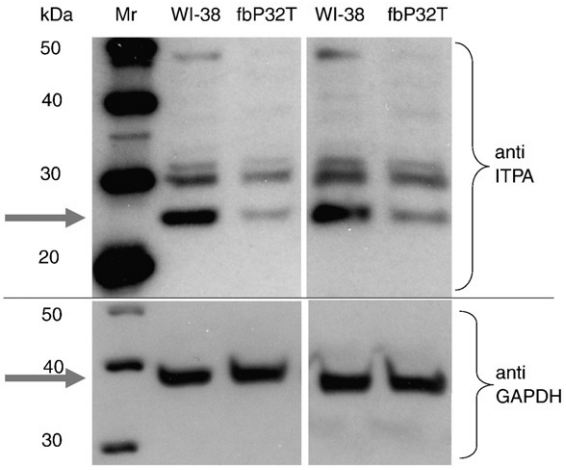

Fig. 6. Diminished levels of P32T ITPA in human fibroblasts. Top blot represents the results of two independent Western blots of extracts of human fibroblasts with anti-ITPA antibodies. Bottom blot serves as a loading control and illustrates that the level of unrelated enzyme, GAPDH, is similar in the two cell lines.

parameters, k_{cat}/K_m connects the reaction rate with the concentration of free enzyme, thus characterizing the *V*-function for low concentrations of substrate. Then, the inhibition is still almost negligible. Thus, we can conclude that ITPA-P32T *in vitro* can effectively catalyze the reaction of pyrophosphohydrolysis, although it is slightly (our estimate is 1.3 times) more sensitive to substrate inhibition by dITP. The ITPA P32T protein is a dimer in solution, such as wild-type ITPA. These observations do not support the hypothesis that the basis of ITPase deficiency in *ITPA-P32T* homozygous patients is in the defect of cross talk between ITPase subunits and the consequent loss of the enzymatic activity.[17] We propose a new, "synthetic" model explaining the *ITPA-P32T* allele phenotypes. Opposite of the idea of "yes or no" effects of ITPA-P32T, we propose that the null phenotype is a result of a catastrophic coincidence of several relatively mild (when alone) changes. The defect of mRNA splicing[34] decreases the amount of normal mRNA available to generate active protein by approximately 50%. In individuals with the mutation, a smaller amount of protein is synthesized in all tissues. Next, the P32T protein is less stable as suggested by the UV thermal denaturation studies and, therefore, might be a more preferable target for proteolytic degradation than the wild-type protein. The efficiency of this degradation could be tissue specific. Indeed, we have found that the level of ITPA in fibroblasts of individual with the 94C→A allele is severely diminished. The enzyme/substrate ratio in blood of such patients is elevated and ITPase is inhibited by substrate. In addition, the P32T protein may be intrinsically more susceptible to substrate inhibition. The net result is a thermally unstable, substrate-inhibited ITPase that is present at such low levels that it is ineffective in protecting patients from purine base analogues. It is also likely that the levels of this unstable but catalytically active P32T ITPA are varying in different tissues from zero (in erythrocytes) to almost normal (presumably in heart). This will explain the discrepancy between the relatively mild effects of the *ITPA-P32T* mutation in humans in comparison to the drastic effects on heart development in *Itpa* knockout mice.[43]

Materials and Methods

E. coli strains and growth conditions

As a starting material, we have used *E. coli* strains AB1157 (used as a wild-type control) and JB30 [Δ*yggV62 recA200*(Ts) *srlC300*::Tn*10*], kindly provided by Dr. A. Kuzminov.[3] The *yggV* is another name of *rdgB*. We modified the strains by introducing the T7 RNA polymerase gene with the Novagen λDE3 Lysogenization Kit. The kit allowed us site-specific integration of λDE3 prophage into an *E. coli* host chromosome. The lysogenized host could be used for controlled expression of target genes cloned in T7 expression pET vectors. The derivative of AB1157 was named EK1 and the derivative of JB30 was named EK5. We have used LB medium supplemented with the appropriate antibiotics to propagate bacteria and Vogel–Bonner minimal medium supplemented with the appropriate nutrients[21] and 0.05 mM IPTG when T7 promoter induction was required.[44]

Plasmid constructs

For the human *ITPA* overexpression in *E. coli* and for purification of the wild-type ITPA, we have used the vector pET15b-ITPA described previously.[44] This plasmid was used for engineering of nucleotide changes encoded for the P32T and E44L changes in ITPA by site-directed mutagenesis (QuikChange kit from Stratagene). To generate the mutations, we have used the following primers (codon sequence encoding the change underlined): for *ITPA-P32T*, P32T-F 5′-GGAGATAAGTTTACC-TGCACTTTGGTGGC and P32T-R 5′-GCCACCAAA-GTGCAGGTAAACTTATCTCC; for *ITPA-E44L*, E44L-F 5′-CAGAAAATTGACCTGCCGTTGTACCAGGGG-GAGCCG and E44L-R 5′-CGGCTCCCCCTGGTA-CAACGGCAGGTCAATTTTCTG.

All results of site-directed mutagenesis were verified by DNA sequencing using the primers ITPA_sN 5′-TCATTGGTGGGGAAGAAGATC and ITPA-sC 5′-AAGCTGCCAAACTGCCAAA. To study the effects of *ITPA* expression in yeast, we cloned the *ITPA* gene and its mutant alleles amplified from corresponding pET15b vectors into XhoI–HindIII sites of the yeast expression vector pESC-URA (Stratagene). This generated N-terminal fusion of the *ITPA* with the sequence encoding for the c-*myc* epitope and put the expression of the resulting gene under the *GAL1* promoter. The yeast *HAM1* gene has been cloned into the pESC-URA vector at the BglII–SacI restriction sites, generating N-terminal fusion of the *HAM1* with the sequence encoding for the FLAG epitope under the *GAL10* promoter.

ITPA protein purification

The genes encoding for recombinant 6-His-tagged human wild-type ITPA and its mutant forms (with substitutions P32T and E44L) were expressed in the *E.*

coli Rosetta2(DE3) strain (Novagen) and purified by affinity chromatography as described previously.[44] To check the purity of extracted proteins, we loaded samples of the peak fraction from a nickel column on SDS-PAGE (Fig. 1b). Purity of all proteins was approximately 95%. The average yield of three protein variants was the same, about 5 mg of protein from 1 g of bacteria. The protein from peak fractions was dialyzed against BICINE–NaCl buffer and stored at −20 °C in a similar buffer with glycerol and DTT [50% glycerol, 20 mM BICINE (pH 8.5), 5 mM DTT, 50 mM NaCl, and 1 mM ethylenediaminetetraacetic acid]. These samples were used for studies of enzymatic activity. For biophysical characterization of ITPA, the His tag was cleaved and the enzyme was further purified by ion-exchange chromatography as described.[44]

Protein activity assay

To measure enzymatic constants for purified proteins, we determined the amount of inorganic pyrophosphate released at different concentrations of natural substrates (ITP or dITP). First, we performed the enzymatic reaction in the reaction mix (10 μL) containing 50 mM Tris–HCl (pH 9), 1 mM DTT, 10 mM $MgCl_2$, varying concentrations of substrate (0.05–5 mM), and 15 nM ITPA. We added the enzyme to a pre-warmed mixture and incubated it for 5 more minutes at 37 °C. Reactions were stopped by adding 1 μL of 10 mM ethylenediaminetetraacetic acid. To measure the concentration of the released pyrophosphate, we used 4 μL of reaction mixture and the P_iPer™ Pyrophosphate Assay Kit (Invitrogen). ITPA is sensitive to strong substrate inhibition. Therefore, we used special equations[59] to determine kinetic parameters:

$$V = V_{max} \times S/\left(K_s + S + S^2/K'_s\right) \quad (1)$$

where V_{max} is the maximum velocity of the enzymatic reaction, S is substrate concentration, K_s is a constant for the formation of active enzyme complex, and K'_s is a constant of the formation of an inactive complex.

$$K_m = V_{max}/V_o \quad (2)$$

where K_m is the Michaelis constant and V_o is the initial velocity of the enzymatic reaction.

First, we solved Eq. (1) to determine the V_{max} value. To solve this equation for three unknown parameters, we compiled a special program in the MatLab system (MatLab 6.5, The MathWorks, Inc.). The program simultaneously uses the four groups of values with three values of substrate concentrations and reaction velocity in each group. Then we used the V_{max} value to determine K_m via Eq. (2). The V_o value was calculated as a derivative of the V-function for $S=0$.

Size-exclusion chromatography–multi-angle light scattering

Dynamic light scattering helps the investigator understand the size distribution, stability, and aggregation state of macromolecules in solution.[60] Here we used a more sophisticated procedure to examine the molecular weight of ITPA molecules, which involves size-exclusion chromatography and analysis of resulting fractions by multi-angle static light scattering[61] to determine absolute molecular weight of the proteins in solution. SEC–MALS experiments were performed by loading 100 μL of protein at the indicated concentrations onto a Superose 6 10/300 GL column (Amersham Biosciences) with a bed volume of approximately 24 mL, an exclusion limit of 4×10^4 kDa, and an optimal separation range of 5 to 5×10^3 kDa. Protein concentrations were determined by measuring the absorption at 280 nm with a NanoDrop® ND-1000 Spectrophotometer, blanked with elution buffer, and using a molar extinction coefficient of 19,900 M^{-1} cm^{-1} and a molecular weight of 21.73 kDa as determined by DNASTAR software. A flow rate of 0.3 mL/min was maintained with an Agilent HPLC instrument. The column was eluted with 20 mM BICINE (pH 8.5), 20 mM NaCl, 2 mM β-mercaptoethanol. Downstream from the column, a UV detector (Agilent), a miniDAWN triple-angle light scattering detector (Wyatt Technology), and an Optilab DSP interferometric refractometer (Wyatt Technology) were connected in series. The refractometer provided a continuous index of protein concentration. A dn/dc (specific refractive index increment) value of 0.185 mL/mg was used. Bovine serum albumin was used as an isotropic scatterer for detector normalization. The intensity of light scattered by a protein is directly proportional to its weight-average molecular mass and concentration. Therefore, molecular masses (M_W) were calculated from light scattering and interferometric refractometer concentration data using ASTRA software. Fractions (0.1 mL) were collected and analyzed by SDS-PAGE followed by silver staining.

UV denaturation curves of ITPA

The purified protein with cleaved His tag[39] was concentrated using an Amicon Ultra 5MWCO spin concentrator (Millipore) and adjusted to 2.2–2.7 mg/mL, then examined using dynamic light scattering (DynaPro MS/X, Protein Solutions, Inc.) to confirm monomodality and monodispersity before the UV denaturation experiments. Preparations showing bimodal peaks were further polished by use of a size-exclusion column (Superdex 200, GE Healthcare), rechecked for monomodality, and reconcentrated as above. UV temperature-induced denaturation curves of ITPA were performed using a Perkin Elmer UV–Vis Spectrophotometer Lambda10. Refining the UV denaturation curve data required several experiments involving a range of protein concentrations. To achieve this without exceeding the detection limits of the spectrophotometer, we employed cuvettes of varied light path lengths (1, 2, 5, and 10 mm), which allowed up to a 10-fold concentration change while maintaining the same A_{280} (≈0.4). The samples were loaded into these cuvettes and all were assayed at 35–65 °C with a heating rate of 0.2 °C/min. Response was measured and recorded every 0.2 s at 280-nm absorbance.

The data were smoothed using a Savitzky–Golay filter, implemented in the freely available MacroBundle for Microsoft Excel† and plotted as A_{280} *versus* temperature. Plots of the first derivative with respect to temperature were generated with the same software. Melting temperatures, T_Ms, were determined by taking the temperature at the maximum of the first-derivative plots.

Antibodies against ITPA

We have sent 4 mg of pure ITPA with His tag cleaved to Rockland Biosciences. Two rabbits were immunized twice at their facility; blood was drawn and the serum was sent

† http://www.bowdoin.edu/~rdelevie/excellaneous/

back to us. The serum was run on Protein G resin at the UNMC Monoclonal Antibody Facility to yield pure polyclonal antibody against ITPA.

Temperature sensitivity test in *E. coli*

The cultures of transformants of the temperature-sensitive strain EK5 (see above) by pET15b vector with wild-type or mutated *ITPA* were grown in LB at a permissive temperature (30 °C). Serial dilutions were spotted on IPTG-containing plates and plates were incubated at 30 or 42 °C (nonpermissive) overnight.

HAP mutagenesis test in bacteria

We performed a spot test by replica-plating of VB plates with growing cells and spotted HAP onto LB plates with rifampicin as described.[22]

HAP sensitivity assay in yeast

We have used yeast strain yo699,[62] which was modified as follows. First, it was made Ade^+ by transformation with a PCR fragment of the *ADE2* gene. Second, we introduced the disruption of the *HAM1* gene by one-step disruption with the DNA fragment released from HLAM plasmid.[19] The strain was transformed by the pESC-URA vector with human *ITPA* and its alleles to Ura^+. Individual, independent transformants were grown in synthetic medium without uracil. Forward Can^r mutant frequencies in the presence of HAP were determined as described previously.[63]

Human cell culture

The normal diploid human lung fibroblast cell line, WI-38 (ATCC CCL-75), was kindly provided by Dr. Vera Gorbunova (University of Rochester, NY). The human fibroblast cell line, abbreviated as fbP32T, which is homozygous for a C→A transversion at nucleotide 94 (94C→A) in exon 2 of the *ITPA* gene, resulting in a proline-to-threonine substitution at codon 32 (P32T), was obtained from the Coriell Institute Biorepository (GMO1617). According to the description provided by the vendor, the patient who donated the cell line is a 29-year-old Caucasian female homozygous for the 94C→A mutation resulting in no detectable ITPA activity in red blood cells and 20% of normal ITPase activity in lymphoblasts. We confirmed the presence of this DNA sequence change by sequencing of appropriate PCR product and have also detected the accumulation of low level of 10 μmol/L of ITP in the cell line extract, which was not detectable in the control fibroblasts.

These untransformed cell lines were cultivated as monolayers in minimal Eagle's Medium with nonessential amino acids (Invitrogen), containing 10% fetal bovine serum (GIBCO) and the antibiotics penicillin, streptomycin, and gentamicin (Invitrogen) to a total final concentration of 1%. The growth medium was further supplemented with 1 mM sodium pyruvate (Invitrogen). The cells were incubated in a humidified incubator at 37 °C and 5% CO_2. Cells at early passages (<25 passages) were used in all experiments to avoid complications with senescence because WI-38 cells have a mean lifespan of approximately 45 to 60 population doublings.

Western blot analysis

WI-38 and P32T fibroblasts were cultivated as monloayers in 100-mm dishes (5×10^5 cells per plate) until subconfluence. The cells were harvested by washing thrice with phosphate buffered saline, and the pellet was collected by centrifugation. The pellets were resuspended in 100 μL of lysis buffer [phosphate buffered saline (pH 7.4) containing protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN)] and mechanically sheared using a pestle. The lysate was cleared by centrifugation, and protein content determined by the Bradford method. Lysate equivalent to 100 μg of protein was boiled in Laemmli's buffer (Invitrogen) containing β-mercaptoethanol (Sigma-Aldrich). The protein samples were resolved on a 10–20% Tris–glycine gel (Invitrogen) by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked overnight in commercially available blocking buffer. Membranes were then incubated in 1:500 dilutions of primary antibody against ITPA (described above) and GAPDH (Cell Signalling, #2118) for 1 h at room temperature. The membranes were then washed with commercially available washing buffer (Thermo Scientific) five times for 10 min each. This was followed by incubation with secondary antibody (1:1500 dilution; Cell Signaling, #7074) for 40 min at room temperature. This was followed by washing (five times for 10 min each) and detection by an ECL system (Thermo Scientific) according to the manufacturer's instructions. Images on film were scanned and the resulting TIFF files were analyzed using Image Quant TL software.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.07.051

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