A Mutation in the Catalytic Subunit of cAMP-Dependent Protein Kinase That Disrupts Regulation

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A mutant catalytic subunit of adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase has been isolated from *Saccharomyces cerevisiae* that is no longer subject to regulation yet retains its catalytic activity. Biochemical analysis of the mutant subunit indicates a 100-fold decreased affinity for the regulatory subunit. The mutant catalytic subunit exhibits approximately a threefold increase in Michaelis constant for adenosine triphosphate and peptide cosubstrates, and is essentially unchanged in its catalytic rate. The nucleotide sequence of the mutant gene contains a single nucleotide change resulting in a threonine-to-alanine substitution at amino acid 241. This residue is conserved in other serine-threonine protein kinases. These results identify this threonine as an important contact between catalytic and regulatory subunits but only a minor contact in substrate recognition.

PROTEIN KINASES PLAY AN IMPORTANT role in regulating cellular processes (*1*). Structural analysis of a number of these enzymes has identified separate catalytic and regulatory domains. The function of the regulatory domain is to inhibit kinase activity. These domains can be on separate subunits, as in adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (cAdPK) (*2, 3*), or within the same polypeptide, as in cyclic guanosine 3′,5′-monophosphate (cGMP)–dependent protein kinase (cGdPK) (*4*), protein kinase C (PK-C) (*5*), and myosin light chain kinase (*6*). It has been postulated (*6, 7*) that inhibition of phosphotransferase activity is maintained by occupation of the active site of the catalytic domain by a pseudosubstrate sequence within the regulatory domain. The association between regulatory and catalytic domains has been shown to be extremely tight, with binding constants in the subnanomolar range (*3*). Activation of these enzymes occurs by dissociation of the regulatory domain and is triggered by binding of small effectors (cAMP, cGMP, Ca^{2+}, and phospholipid) or activating proteins (calmodulin) to the regulatory domain (*3*). Biochemical studies with synthetic peptides have focused on delineating residues in the regulatory domain that comprise the pseudosubstrate sequence (*6, 7*). We are interested in identifying amino acid residues in the catalytic domain that stabilize the interactions between the regulatory and catalytic domains in protein kinases. cAMP-dependent protein kinase was studied because it is the best characterized protein kinase.

In *Saccharomyces cerevisiae*, as in higher eukaryotes, the holoenzyme of cAdPK is a tetrameric molecule comprised of two catalytic (C) and two regulatory (R) subunits (*2, 8*). In the absence of cAMP, the R subunit binds tightly to the C subunit and inhibits catalytic activity. The effector cAMP binds to the R subunit, and the holoenzyme dissociates into two active C subunits and an R subunit dimer. The C subunit phosphorylates specific protein substrates, thereby altering the biochemical properties of these proteins (*1*). In yeast, three genes, *TPK1*, *TPK2*, and *TPK3*, encode distinct catalytic subunits, C_1, C_2, and C_3, respectively (*9*). All three proteins are structurally and functionally homologous with the mammalian isozymes (*9*). A single gene, *BCY1*, encodes the yeast regulatory subunit (*10*). Yeast R subunit is homologous to mammalian R subunits, and it resembles the mammalian type II R subunit in its ability to act as a substrate for C (*8, 11*).

Synthesis of cAMP in *S. cerevisiae* is catalyzed by adenylate cyclase (*CYR1*) and is positively regulated by the *RAS1* and *RAS2* gene products (*12, 13*). At least one of the *RAS* genes is normally required for viability, but both can be deleted in strains that lack a functional R subunit of cAdPK (*12*). On the basis of these studies, we predicted that mutations in C subunit that disrupt regulation of catalytic activity would suppress a ras^{ts} defect. We isolated a dominant suppressor of a temperature-sensitive allele of *RAS2* that mapped to the *TPK1* gene (*14*). DNA sequencing of the coding region of the mutant gene (*15*) identified a single adenine-to-guanine transition at nucleotide number 721, resulting in a threonine-to-alanine substitution at amino acid 241 of C_1 (*16*). This residue is conserved between the yeast and mammalian catalytic subunits (*17*). The analogous threonine in mammalian C subunit is phosphorylated (*17*). It is not yet known whether Thr^{241} is phosphorylated in yeast C_1. Studies on mammalian C subunit suggest that a neighboring residue is in close proximity to the protein substrate binding site (*18*).

To compare the biochemical properties of wild-type and mutant C_1, we constructed yeast strains that overexpress only wild-type C_1 or $C_1(Ala^{241})$ (*19*). Soluble extracts from the overexpressing strains were subjected to gel filtration chromatography. The kinase activity from the wild-type strain was entirely cAMP-dependent and eluted as a tetramer from the sizing column. The activity from the $C_1(Ala^{241})$ strain was cAMP-independent with a molecular size consistent with monomer C_1 subunit (*20*). Wild-type and mutant catalytic subunits were purified (*21*), and their affinities for R subunit and kinetic parameters were determined. Increasing amounts of purified R subunit added to wild-type C_1 results in inhibition of catalytic activity with an IC_{50} (*22*) of 63 n*M* (Fig. 1A). In contrast, $C_1(Ala^{241})$ shows no loss of kinase activity with the addition of up to 1.6 μ*M* R. Since we were unable to quantitate the difference in affinity for wild-type R, we examined the inhibition of mutant and

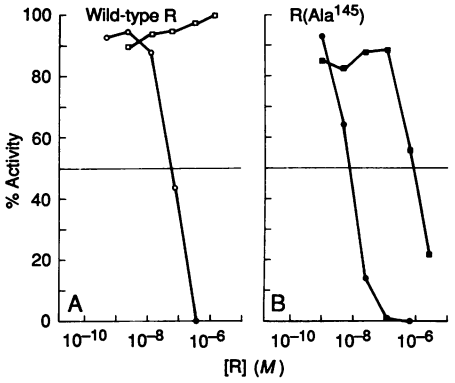

Fig. 1. Inhibition of purified wild-type C_1 (○, ●) and mutant $C_1(Ala^{241})$ (□, ■) proteins by R subunit. C subunit (1 n*M*) was preincubated with increasing concentrations of either (**A**) wild-type R subunit or (**B**) mutant $R(Ala^{145})$ subunit in the presence of 200 μ*M*[γ-^{32}P]ATP (200 counts per minute per picomole), 50 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MOPS) (*p*H 6.5), 10 m*M* $MgCl_2$, and bovine serum albumin (100 μg/ml) (total volume 45 μl) for 5 minutes at 30°C. The kinase assay was initiated by the addition of 5 μl of 1 m*M* Kemptide to the preincubation mixture. After 10 minutes at 30°C, one-half of the reaction mixture was spotted onto Whatman phosphocellulose (P-81) paper and washed for 20 minutes in four changes of 75 m*M* phosphoric acid (*25*). Strips were air dried and counted in scintillation fluid (Aquasol-2). Residual kinase activity was measured and expressed as a percentage of the activity of C subunit in the absence of R subunit. Data points reflect averages of duplicate assays performed on a single sample. Yeast wild-type R and $R(Ala^{145})$ were purified from expression strains of *Escherichia coli* as described by Johnson *et al.* (*11*).

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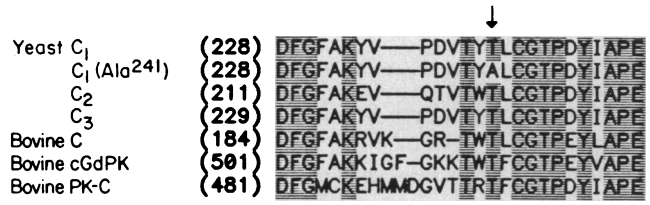

Fig. 2. Amino acid sequences of the mutant, C_1(Ala^{241}), and wild-type yeast C_1 subunits and other serine-threonine protein kinases in the region of the putative substrate-binding domain. Shading indicates absolutely conserved amino acids. The arrow points to the site of the substitution in C_1(Ala^{241}). Numbers in parentheses indicate the first amino acid listed. Related protein kinases are the other two isozymes of the yeast C subunit (C_2 and C_3) (*9*), the C_α subunit of the bovine cAMP-dependent protein kinase (bovine C) (*17*), the bovine cGMP-dependent protein kinase (cGdPK) (*4*), and bovine protein kinase C_α (PK-C) (*5*). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; T, Thr; V, Val; W, Trp; and Y, Tyr.

Table 1. Kinetic comparison of C_1(Ala^{241}) and C_1 proteins. C_1(Ala^{241}) and pure C_1 proteins were assayed by varying the concentrations of ATP or Kemptide. K_m and k_{cat} values for ATP and for substrate (Kemptide) were determined from Eadie-Hofstee plots (*26*) by linear regression analysis. Data points reflect averages from duplicate assays of a single sample all of which agreed within ±10%. Values given are ±95% confidence interval. Since the mutant C_1(Ala^{241}) is only approximately 30% pure, k_{cat} values for ATP and Kemptide were calculated from the maximum velocity, and an estimated protein concentration was made by comparison with known concentrations of C_1 on a protein gel stained with Coomassie blue. IC_{50} values (*22*) for R and R(Ala^{145}) were determined from Fig. 1.

Protein	K_m (μM)	k_{cat} (minute^{-1})	k_{cat}/K_m ($\times 10^6$ min^{-1} M^{-1})	IC_{50} [R] (nM)	IC_{50} [R(Ala^{145})] (nM)
			Kemptide		
C_1	115 ± 9	600 ± 26	5.22 ± 0.39	63.1	7.1
C_1(Ala^{241})	424 ± 42	717 ± 54	1.69 ± 0.13	>>1600*	790
			ATP		
C_1	27.1 ± 3.7	552 ± 63	20.4 ± 2.4		
C_1(Ala^{241})	71.3 ± 26.7	572 ± 157	8.02 ± 2.37		

*No inhibition of C_1(Ala^{241}) activity was seen with the addition of up to 1.6 μM R.

wild-type kinase activities using a modified R subunit with a tenfold higher affinity for wild-type C_1 (*23*). This modified R subunit was constructed by site-directed mutagenesis, replacing the serine at position 145 with alanine. This R(Ala^{145}) (*24*) inhibits wild-type C_1 with an IC_{50} of 7.1 nM and inhibits C_1(Ala^{241}) with an IC_{50} of 790 nM (Fig. 1B and Table 1). These data indicate that C_1(Ala^{241}) exhibits at least a 100-fold decrease in affinity for R compared to wild-type C_1. The Thr^{241}-to-Ala substitution does not significantly affect catalytic activity (Table 1). The substitution causes a 2.5-fold increase in the apparent Michaelis constant (K_m) for adenosine triphosphate (ATP) and a 3.5-fold increase in the apparent K_m for a peptide substrate (Kemptide). The catalytic rates (k_{cat}) remain essentially unchanged.

Biochemical experiments demonstrate that Thr^{241} of yeast C_1 is important for association with the regulatory subunit but plays only a minor role in substrate recognition. This conclusion is confirmed by genetic evidence. Toda *et al.* demonstrated that at least one functional *TPK* gene is required for viability (*9*). The strain that we used for the purification of the mutant protein contains C_1(Ala^{241}) as the only C subunit of cAdPK (*21*). Therefore, C_1(Ala^{241}) can substitute for the necessary in vivo functions of the C subunit. This is the first example of a mutation in the catalytic subunit that affects R-C interaction. This mutant protein C_1(Ala^{241}) will be an important reagent for studying the role of cAMP-dependent protein kinase in specific tissues by introduction of the gene encoding the mutant C subunit into different cell lines. This offers a more powerful approach than activation of cAMP-dependent protein kinase through stimulation of adenylate cyclase by drugs or hormones.

A number of other amino acids including Thr^{241} in the region from Asp^{228} to Glu^{252} are conserved in mammalian cAdPK, cGdPK, and PK-C (Fig. 2). As discussed above, these protein kinases are composed of separate regulatory and catalytic domains and may be regulated by pseudosubstrate inhibition. The sequence conservation and apparent common regulatory mechanism of these protein kinases suggest that the functional role of this threonine is conserved.

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14. Spontaneous suppressors were selected at the non-permissive temperature (37°C) from yeast strain STS8 (MATα *ras2*^{ts8} *ras1*::*URA3 his3 ade8 trp1 leu2 ura3*). Dominant suppressors were identified by mating to strain LL4-1B (MATα *ras2*^{ts8} *ras1*::*HIS3 his3 ade8 trp1 leu2 ura3*) and testing the resulting diploids for temperature sensitivity. Thirty out of 221 suppressors were dominant as judged by this assay. Strains containing a mutant C subunit were selected from among the dominant suppressors by disrupting each *TPK* gene individually and retesting for temperature sensitivity. Haploid strains containing dominant suppressors were transformed with DNA fragments designed to disrupt *TPK1*, *TPK2*, or *TPK3* [R. J. Rothstein, *Methods Enzymol.* **101**, 202 (1983)]. Transformants were tested for restoration of the temperature-sensitive phenotype. Yeast strain STR6, which by genetic analysis contained a single dominant suppressor gene, regained its temperature sensitivity upon disruption of *TPK1*, indicating an activating mutation in this gene. The mutant *TPK1-str6* gene was isolated by homologous recombination (T. L. Orr-Weaver, J. W. Szostak, R. J. Rothstein, *ibid.*, p. 228) into a linearized retrieval plasmid (pKRP-1) containing *TPK1* flanking sequences, an M13 origin of replication [J. Viera and J. Messing, *ibid.* **153**, 3 (1987)], and yeast–*Escherichia coli* shuttle sequences. Yeast DNA from strains that received pKRP-1 was used to transform *E. coli* to ampicillin resistance. Clones containing the recombined *TPK1* gene were identified by colony hybridization with a *TPK1*-specific probe. Confirmation that the mutant *TPK1-str6* gene was responsible for suppression of the *ras2*^{ts} mutation was accomplished by showing that replacement of *TPK1* by *TPK1-str6* could suppress the temperature sensitivity of strain LL4-1B.
15. The DNA sequence of the entire coding region of the mutant *TPK1-str6* gene was determined by dideoxy nucleotide sequencing [F. Sanger, A. R. Coulson, B. G. Barrel, A. J. H. Smith, B. A. Roe, *J. Mol. Biol.* **143**, 161 (1980)] with the use of six synthetic oligonucleotide primers.
16. Nucleotide and amino acid number are from Toda *et al.* (*9*).
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18. Cys^{199} in the mammalian C subunit (analogous to Cys^{243} in C_1) is protected from iodoacetic acid alkylation by R subunit [N. C. Nelson and S. S. Taylor, *J. Biol. Chem.* **258**, 10981 (1983)] and can form a disulfide bridge with synthetic peptide substrate analogs [H. N. Bramson *et al.*, *ibid.* **257**, 10575 (1982)].
19. C_1(Ala^{241}) is the mutant catalytic subunit of yeast cAdPK that contains alanine at position 241.
20. L. R. Levin and M. J. Zoller, unpublished observation.
21. Wild-type C_1 was purified as described [M. J. Zoller, J. Kuret, S. Cameron, L. R. Levin, K. E. Johnson, *J. Biol. Chem.*, in press]. C_1(Ala^{241}) was purified from strain LL2A-1 [MATα *his3 leu2 ura3 trp1 ade8 tpk1*::*URA3 tpk2*::*HIS3 tpk3*::*TRP1* YEp(*LEU2*)-*TPK1-str6* YEp(*ADE8*)*BCY1-ala145*] by the same method. This strain contains null alleles of each of the wild-type *TPK* genes and overexpresses the mutant *TPK1-str6* and *BCY1-ala145* genes on yeast multicopy plasmids (YEp). The R subunit mutant, *BCY1-ala145* (J. Kuret, K. E. Johnson, C. Nicolette, M. J. Zoller, *ibid.*, in press), was constructed by oligonucleotide mutagenesis [M. J. Zoller and M. Smith, *DNA* **3**, 479 (1984)] and overexpressed with C_1(Ala^{241}) to induce formation of the holoenzyme.

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27. We thank M. Ockler and J. Duffy for artwork and D. Greene for photography. Supported by NIH grants GM33986 (M.J.Z.) and R35 CA39829-02 (M.W.), the American Cancer Society (M.W.), the American Business Foundation for Cancer Research, and Pfizer Biomedical Research Award.

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Tat Protein from Human Immunodeficiency Virus Forms a Metal-Linked Dimer

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Tat, the transactivating protein from HIV, forms a metal-linked dimer with metal ions bridging cysteine-rich regions from each monomer. This novel arrangement is distinct from the "zinc finger" domain observed in other eukaryotic regulatory proteins. Ultraviolet absorption spectra show that Tat binds two Zn^{2+} or two Cd^{2+} ions per monomer, and electrophoresis of the Tat-metal complexes demonstrates that the protein forms metal-linked dimers. Partial proteolysis and circular dichroism spectra suggest that metal binding has its primary effects in the cysteine-rich region and relatively little effect on the folding of other regions. These results suggest new directions for biological studies and new approaches to drug design.

THE HUMAN IMMUNODEFICIENCY virus (HIV) encodes several regulatory proteins that are not found in simpler retroviruses. The Tat protein, which is one of these regulatory proteins, transactivates genes that are expressed from the HIV long terminal repeat (LTR) (*1*) and Tat is essential for viral replication in vitro (*2, 3*). The mechanism of transactivation is unclear. Tat seems to cause accumulation of messenger RNA (mRNA) by acting as a transcriptional anti-terminator (*4*), by increasing the rate of transcription from the LTR promoter (*5*), or by stabilizing viral mRNAs (*6–9*). Tat may also increase translational efficiency (*6–11*). Deletion analysis has shown that a region of 50 to 100 base pairs near the 3′ end of the LTR is required for transactivation by Tat (*12*). RNA from this site (which is called the TAR site) forms two stable stem-loop structures, and it has been suggested that these double-stranded regions may be binding sites for Tat (*9, 13, 14*).

The Tat protein contains 86 amino acids (*15, 16*). Its sequence includes a highly basic region (two lysines and six arginines within nine residues) that might participate in nucleic acid binding. Tat also contains a cysteine-rich region (seven cysteines within 16 residues), and sequence comparisons from several HIV isolates show that the cysteines are perfectly conserved. Genetic studies have shown that fragments of Tat are biologically active (*8, 9, 16, 17*), but each of the active fragments (1 to 72 as a protein and 1 to 56 or 1 to 58 as fusion proteins) retains both the cysteine-rich region and the basic region. The cysteine-rich region is strikingly similar to the metallothioneins (*18, 19*) and also matches a test filter that was devised to locate potential metal-binding sites (*20*). Although this filter has often been used to identify possible "zinc finger" domains, the high density of cysteines in Tat suggested that this protein might have a novel structure. We show that Tat forms a metal-linked dimer and that its structure is distinct from the zinc finger motif found in other eukaryotic regulatory proteins.

Tat has been overexpressed in *Escherichia coli* (*21*), and protoplast fusion experiments show that the protein produced in *E. coli* allows a virus deficient in the Tat protein to replicate (*2*). On SDS gels, this protein migrates as a 15-kD band and thus is indistinguishable from Tat expressed in mouse cells or HIV-infected cells (*7, 21*). The *E. coli* cells induced for Tat expression (*22*) were sonicated in lysis buffer (50 m*M* tris-HCl, *p*H 8.0, 200 m*M* KCl, 2 m*M* EDTA) and the initial lysate was cleared by centrifugation. Polyethyleneimine was added to 0.5%; the mixture was stirred at 4°C for 30 minutes and then centrifuged. This pellet was resuspended in lysis buffer containing 700 m*M* KCl (which was required to resolubilize Tat). Protein was precipitated again with 40% ammonium sulfate, and the pellet was resuspended in a small volume of S-Sepharose washing buffer (20 m*M* tris-HCl, *p*H 7.5, 50 m*M* KCl, 2 m*M* EDTA). This material was placed on a S-Sepharose column and eluted with a 0.05*M* to 1.0*M* KCl gradient. Tat was tentatively identified by SDS gel electrophoresis as a protein with an apparent molecular size of 15,000 daltons, and it eluted as a very broad peak at 0.3 to 0.6*M* KCl. The fractions containing Tat were pooled, concentrated by pressure filtration, and treated with 0.5*M* dithiothreitol in 6*M* guanidine hydrochloride. The reduced protein was loaded onto a C_4 reversed-phase high-performance liquid chromatography (HPLC) column in 0.1% trifluoroacetic acid and eluted with an acetonitrile gradient. The Tat protein eluted with approximately 32% acetonitrile and was immediately lyophilized. Western blotting of the SDS gels, with a previously described antibody (*21*), confirmed that the purified protein was Tat. Amino acid analysis of the purified sample also confirmed the identity of Tat and allowed us to quantitate the amount of protein. Tat has an absorption maximum at 278 nm with an extinction coefficient of 1.32 optical density units per milligram of pure protein in 20 m*M* tris-HCl, *p*H 7.2.

To study the metal binding of Tat, we needed to ensure that the protein was reduced. After HPLC purification and lyophilization, Tat was resuspended in water and reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (*23*) indicated the presence of 7 mol of free thiol per mole of Tat. Careful handling was required to prevent reoxidation. Under nonanaerobic or nonreducing conditions, Tat oxidized to form disulfide-linked multimers that could be seen by gel electrophoresis (see below). To prevent oxidation, we carried out all manipulations in an anaerobic chamber, and all solutions were degassed with helium and equilibrated in the chamber before use.

Optical absorption spectra gave the first evidence that Tat binds metals. The spectra obtained with reduced Tat and 0 to 2.0 molar equivalents of $CdCl_2$ (Fig. 1A) show that the absorbance increases as Cd^{2+} is added. There is a maximum at 248 nm in the difference spectra, indicating charge transfer transitions between the metals and sulfur ligands. Addition of more than two equivalents of $CdCl_2$ did not further change the spectrum, suggesting that two Cd^{2+} ions bind to the protein. Titrating the Tat-Cd^{2+} complex with acid showed that the metals must be tightly bound because the complex remained stable until the *p*H fell below 4.0 (Fig. 1B). No metal binding to oxidized Tat was observed. Ultraviolet absorption spectra were also used to monitor the binding of

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