

Identification of Functionally Distinct Regions That Mediate Biological Activity of the Protein Kinase A Homolog Tpk2^{*[S]}

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Kinases regulate key signaling processes that are increasingly implicated in development and disease. Kinase modulators have become important therapeutic tools and often target catalytic domains that are among the most structurally and functionally conserved regions of these enzymes. Such therapies lose efficacy as mutations conferring resistance arise. Because interactions between distinct and often distant regions of kinases can be critical, we took an unbiased genetic approach to identify sites within the protein kinase A homolog Tpk2 that contribute to its biological activity. Because many of these map outside the conserved core, this approach should be broadly useful in identifying new, more kinase-specific therapeutic targets.

Genes encoding protein kinases constitute one of the largest gene families and encompass ~2% of both yeast and human genomes (1). The protein kinase superfamily is classified by its catalytic activity; that is, transferring the γ -phosphate from ATP to a substrate. Kinases phosphorylate proteins and other metabolites to regulate their activity. Indeed, post-translational phosphorylation is a chief mechanism for intracellular cell signaling. Phosphate is such a ubiquitous tag for intracellular signaling that roughly one-third of all cellular proteins are phosphorylated at any moment (2). Kinase activity is essential for signal transduction, and misregulation or altered kinase activity is associated with a myriad of diseases, including many cancers, Alzheimer disease, arthritis, Parkinson disease, and diabetes (3–5).

Kinases have evolved to differ in size, specificity, and mechanism of regulation of activity. However, the kinase superfamily shares a highly conserved core containing several invariant amino acids that are critical for activity *in vitro* (6). Although this kinase core is conserved among all metazoans, it is frequently flanked by N-terminal and C-terminal extensions that contribute at multiple levels to substrate specificity (7).

Among the kinases, cAMP-dependent protein kinase A (PKA)⁴ has been intensively studied structurally and enzymatically and serves as a prototype for the superfamily (8–10). PKA is a global eukaryotic regulator of cellular processes, including control of division, metabolism, differentiation, and apoptosis. PKA also links external and internal cellular environments; external hormonal stimuli that change intracellular cyclic adenosine 5'-monophosphate levels (cAMP) modulate kinase activity.

The activity of PKA is regulated through interactions with its regulatory subunit, Bcy1. Two monomeric units of PKA interact with a Bcy1 dimer during an inhibited state. When intracellular cAMP levels are increased, each regulatory subunit binds two molecules of cAMP, causing a conformational change whereby the catalytic subunits of PKA are released and subsequently activated (11, 12).

Structurally, members of the PKA family share the highly conserved kinase core that consists of small and large lobes separated by a nucleotide binding pocket (Fig. 1A). The small lobe contains the C-helix that makes direct contacts with the nucleotide and is part of the nucleotide binding pocket. Also in this lobe is the glycine-rich loop that binds to ATP and positions the γ -phosphate of ATP for phosphoryl transfer (13). A linker region between residues 120–127 tethers the small and large lobes and acts to dictate motion between the two lobes. The large lobe contains many residues in proximity to the nucleotide binding pocket that are highly conserved and are directly involved in catalysis, including the catalytic loop, the magnesium positioning loop, and the activation loop (14).

In *Saccharomyces cerevisiae* PKA enzymes are encoded by three genes: *TPK1*, *TPK2*, and *TPK3* (15, 16). No individual *TPK* gene is essential in yeast, but at least one gene must be intact for viability (15), demonstrating that essential substrates may be acted upon by all three isozymes. However, when two of the three *TPK* genes are mutated, distinct phenotypes have been observed, establishing that unique substrates for each gene product may contribute to distinct signaling responses (17–20). Of the three genes, loss of *TPK2* results in some of the strongest

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⁴ The abbreviations used are: PKA, protein kinase A; FOA, 5-fluoroorotic acid.

phenotypes, including effects on pseudohyphal growth, respiratory growth, transcriptional regulation, and utilization of alternative carbon sources (18, 19, 21, 22).

Each Tpk protein has a highly conserved kinase core that is nearly identical among the three. Some specific kinase functions, such as pseudohyphal development, map to the core domain (23). However, each protein also contains N-terminal and C-terminal extensions flanking the core. Although the C-terminal extensions are highly conserved among the PKA superfamily (24), the N-terminal regions differ greatly in length and sequence among yeast isoforms as well as among other members of the kinase superfamily (15). *TPK2* encodes the shortest N-terminal sequence flanking the kinase core (66 residues *versus* 83 and 84 for *TPK1* and *TPK3*, respectively) yet has the most distinct sequence in this region; that is, two stretches of polyglutamine residues that compose ~30% of this region. Because the three kinases share substantial identity throughout the kinase core and C-terminal extensions, it is likely that their N-terminal regions may be important contributors for cell signaling. Furthering this notion, distinct morphogenetic roles were demonstrated with chimeras created by exchanging N-terminal extensions of *Candida albicans* Tpk proteins (25). The chimeric proteins displayed specific characteristics of the isoforms from which the N-terminal region was derived rather than from the catalytic core and C terminus. These results supported the possibility that specific activity of each isoform of PKA may be at least partially defined by its N-terminal region.

The C-terminal tails are conserved between the three yeast PKA isoforms and among AGC (protein kinase A, protein kinase G, protein kinase C) kinase family members but diverge across the kinase superfamily (26, 27). Within the PKA family, the C-terminal region is a critical regulator of catalytic activity by acting as a molecular gate to limit access to the nucleotide binding pocket (2). This illustrates that kinases have evolved to gain unique activity derived from non-conserved features that promote diversity and substrate specificity.

To evaluate the functional significance of both conserved and non-conserved elements on kinase regulation, a structurally non-biased approach was employed using *S. cerevisiae* Tpk2p as a model. Directed point mutations were first introduced within the conserved nucleotide binding site of Tpk2p. These were screened *in vivo* for their ability to support viability in a *tpk1Δ tpk2Δ tpk3Δ* triple mutant strain. Indeed, subsequent analysis revealed that one mutant even had a dominant negative phenotype, demonstrating that although it could provide essential kinase activity, it additionally affected the activity of wild-type PKA isoforms. One double mutant that failed to support growth or maintain kinase activity was chosen for further analysis. The *tpk2* sequences from the double mutant were mutagenized randomly and used to isolate intragenic suppressors with restored kinase function. These were defined by supporting viability in the *tpk* triple null mutant. Although the suppressors restored viability, many were distinct from wild type because they had clear phenotypes in assays designed to survey cellular functions. Few mutations mapped to sequences encoding the key catalytic residues surrounding the nucleotide binding pocket, yet many mapped to the 3' region of the gene encoding the large lobe of the kinase domain as well as

the unique N-terminal region flanking the kinase core. These results demonstrate that the N- and C-terminal extensions that are unique to Tpk2p can significantly affect kinase activity. Furthermore, both non-conserved regions flanking the core modulate activity *in vivo*, thereby extending a network of catalytic regulation throughout the kinase. Because of the deep functional conservation of many kinases including PKA (28), the experimental approach reported here may provide a basis in defining unique, kinase-specific targets inhibition.

EXPERIMENTAL PROCEDURES

Strains and Media—The *S. cerevisiae* strain used in this study was derived from the *Saccharomyces* Genome Deletion Project (29). The strain, LPY 6292, has the following genotype: *MATα his3-1 leu2-0 lys2-0 met15-0 ura3-0 tpk1Δ::KANMX tpk2Δ::KANMX tpk3Δ::KANMX*. To survive, it carries a *CEN*, *URA3*-marked plasmid bearing wild-type *TPK1* (pLP2024). Where indicated, wild-type and *TPK2* mutants were co-expressed in a *CEN*, *LEU2*-marked vector (30). Growth was at 30 °C unless otherwise specified, and standard techniques for yeast manipulation were performed (31).

Generation of Tpk2p Pocket Mutations and Screening for Restoration of PKA Function—Point mutations were introduced into sequences encoding the nucleotide binding pocket of Tpk2p using the following forward primers the QuikChange site-directed mutagenesis kit (Stratagene) (the corresponding PKA-Cα residue number is listed in parentheses): L76A (49), 5'-ATTATGATAACGGCCGGTACTGGATCT-3'; V84A (57), 5'-TCTTTTGGT-AGAGCTCATTTGGTGCGC-3'; I131A (104), 5'-CATCCGTTTCTCGCGAGAATGTGGGGT-3'; M147A (120), 5'-ATCTTTATGGTGGCCGACTATATCGAA-3'; Y149A (122), 5'-ATGGTGTATGGATGCGATCGAAGGTGGT-3'; L200A (173), 5'-CCAGAAAATATCGCTCTAGATAGAAATGGC-3'; T210V (183), 5'-CACATTAAAATAGTCGACTTTGGGTTCGCC-3'. The double mutants were made sequentially. To isolate intragenic suppressors of the *TPK2-V84A, T210V* mutant, randomly mutagenized libraries were generated starting with the *TPK2-V84A, T210V* allele. Two independent approaches were taken. Libraries were made using either the GeneMorph II random mutagenesis kit (Stratagene) or low fidelity amplification methods to introduce additional mutations throughout the entire coding region. Target template concentrations were varied to alter the mutation frequency to range from 0 to 16 mutations per 1000 base pairs as described in the manufacturer's instructions. The amplified coding sequences were ligated into pRS315 plasmids bearing 2000-bp upstream and 1000-bp downstream flanking sequence of *TPK2* and transformed into *Escherichia coli* DH5α. Colonies were pooled to represent ~250 colonies/culture and grown for ~4 rounds of cell division before DNA preparation. Approximately 25,000 total mutagenized templates were screened for suppression (~100 library pools).

Mutagenized libraries were transformed into the *tpk* null strain and plated onto Leu – Ura – drop-out medium, and double transformants were patched onto Leu – Ura – plates, grown at 30 °C overnight, then replica-plated onto Leu – 5-FOA plates. Plasmids from transformants that retained viability on 5-FOA were rescued into *E. coli*, and the suppressors were

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sequenced (University of California San Diego Cancer Center Sequencing Facility).

Phenotypic Analysis of Strains with Point Mutations in TPK2—Cultures were grown in Leu[−] Ura[−] medium. Cell densities were normalized to $A_{600} = 1.0$ - and 5-fold serially diluted. Dilutions were plated onto selective medium and grown at the indicated temperatures for 6 days. Dilutions were plated onto phenotypic analysis plates that were prepared as previously described (32).

Protein Expression and Immunodetection—Wild-type and mutant TPK2 in the centromeric pRS315 vector were expressed from the endogenous promoter and C-terminal FLAG-tagged. Liquid cultures were grown until late log-phase growth ($A_{600} = 1.0$ – 1.3), and cell lysates were prepared by bead beating in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Sigma-Aldrich) (lysis buffer). Protein concentrations of cell lysates were normalized using Bradford reagent (Bio-Rad), separated on 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Either anti-FLAG (Sigma-Aldrich) or anti-histone H3 K4 (Upstate) was used as the primary antiserum. Horseradish peroxidase-conjugated antisera against mouse or rabbit IgG (Santa Cruz Biotechnology) were used for enhanced chemiluminescent detection (PerkinElmer Life Sciences).

Purification of FLAG-labeled PKA Catalytic Subunit—Cultures expressing wild-type and/or mutant FLAG-tagged versions of TPK2 were grown in Leu[−] Ura[−] medium, pelleted, and resuspended in lysis buffer with protease inhibitor mixture (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride (EMD Chemicals). Cells were lysed by bead beating and incubated with anti-FLAG M2 affinity resin for 4 h at 4 °C. The resin was washed 5 times with lysis buffer, and FLAG-tagged Tpk2p was eluted by competition with FLAG peptide at a final concentration of 150 ng/ μ l.

Catalytic Kinase Activity Assay with Kemptide Substrate—Catalytic activity of wild-type and altered versions of Tpk2p was assayed using the PepTag PKA activity assay kit (Promega). Reactions were set up according to the manufacturer's instructions using either 0.8 or 1.6 μ g of protein per reaction, incubated for 30 min (room temperature), and then stopped by heating at 95 °C for 10 min. The Kemptide substrate is fluorescently labeled, and its overall net charge changes from +1 to −1 upon phosphorylation by Tpk2p. The change in charge was detected by electrophoresis through 0.8% agarose in 50 mM Tris-HCl, pH 8.0, where successfully phosphorylated peptides migrate in the direction opposite to the nonphosphorylated peptides.

RESULTS

The *tpk2-V84A* and *TPK2-V84A,T210V* Alleles Reveal Essential Sites for Tpk2p Function—Because the kinase core is nearly identical between each Tpk protein and mammalian PKA, Tpk2p residues predicted to interact directly with the nucleotide base were identified by sequence alignment (Fig. 1B). Several invariant residues have been identified crystallographically to contribute hydrophobic or H-bond interactions with the nucleotide and were, therefore, predicted to be important for nucleotide binding (8). Several of these had been char-

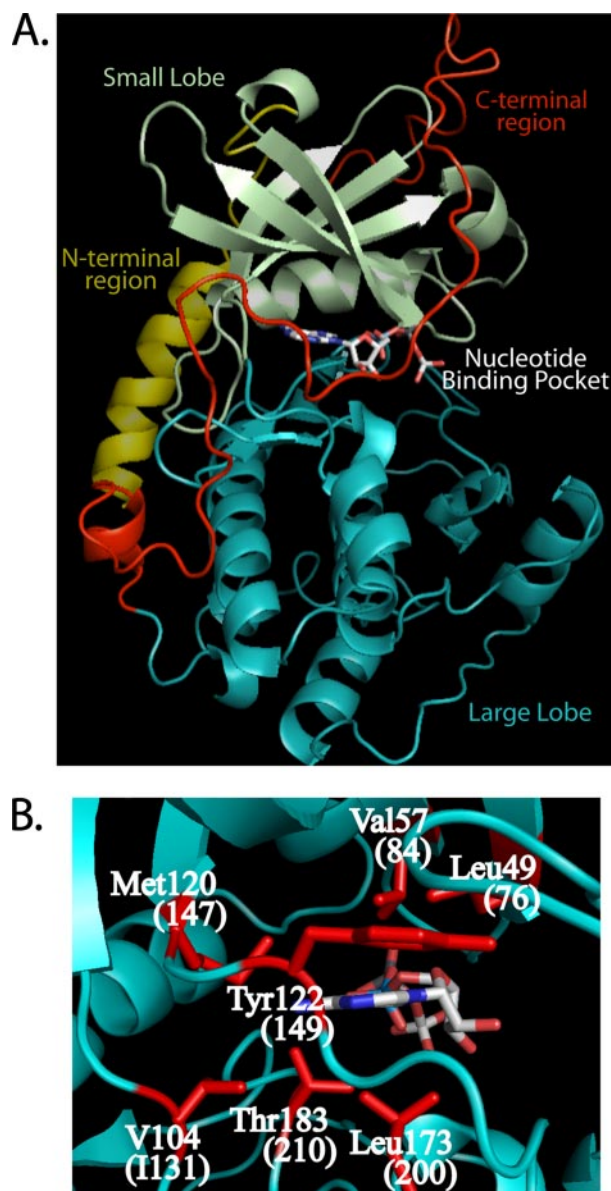


FIGURE 1. Structural features of PKA and mutations introduced within the nucleotide binding pocket. A, a view of the murine isoform of PKA. Major features including the large and small lobes and nucleotide binding pocket are highlighted (58). B, several residues that interact with the nucleotide base in murine PKA lie within the kinase core and include PKA-C α residues Leu-49, Val-57, Val-104, Met-120, Tyr-122, Leu-173, and Thr-183. In Tpk2p, the corresponding residues are Leu-76, Val-84, Ile-131, Met-147, Tyr-149, Leu-200, and Thr-210, in parentheses. These residues are conserved in Tpk2p and are predicted to perform analogous roles in nucleotide binding. The bound nucleotide is represented as a ball-and-stick figure. The structure was adapted from Protein Data Bank code 1ATP and modeled using PyMOL.

acterized previously for *in vitro* effects on catalysis and small molecule binding, but the *in vivo* consequences of many active mutants remains unclear (24, 33). The corresponding sites in TPK2 were individually mutated, and the function of each mutant was assessed in an *S. cerevisiae* strain in which all three genes encoding PKA were deleted (Table 1). Because some PKA activity is essential for viability, this triple null mutant strain was maintained by expression of wild-type TPK1 from a URA3-marked plasmid. We selected this genotype to provide potentially the greatest dynamic range of viability for subsequent analysis because it had been previously demonstrated that

TABLE 1

Wild-type and site-directed mutant *TPK* plasmids used in this study

Plasmid	Description ^a
pLP2024	<i>CEN URA3 TPK1</i>
pLP2025	<i>CEN LEU2 TPK2</i> (224 (ACG→ACC); 324 (TTT→TTC))
pLP2026	<i>pLP2025</i> (L76A (CTT→GCC))
pLP2027	<i>pLP2025</i> (V84A (GTT→GCT))
pLP2028	<i>pLP2025</i> (I131A (ATT→GCG))
pLP2029	<i>pLP2025</i> (M147A (ATG→GCC))
pLP2030	<i>pLP2025</i> (Y149A (TAT→GCG))
pLP2031	<i>pLP2025</i> (L200A (TTG→GCG))
pLP2032	<i>pLP2025</i> (T210V (ACC→GTC))
pLP2033	<i>pLP2025</i> (V84A (GTT→GCT); T210A (ACC→GTC))
pLP2034	<i>CEN LEU2 TPK2::FLAG</i> (224 (ACG→ACC); 324 (TTT→TTC))
pLP2035	<i>pLP2034</i> (L76A (CTT→GCC))
pLP2036	<i>pLP2034</i> (V84A (GTT→GCT))
pLP2037	<i>pLP2034</i> (I131A (ATT→GCG))
pLP2038	<i>pLP2034</i> (M147A (ATG→GCC))
pLP2039	<i>pLP2034</i> (Y149A (TAT→GCG))
pLP2040	<i>pLP2034</i> (L200A (TTG→GCG))
pLP2041	<i>pLP2034</i> (T210V (ACC→GTC))
pLP2042	<i>pLP2034</i> (V84A (GTT→GCT); T210A (ACC→GTC))

^a Note that the description of each plasmid specifies both nucleotide and amino acid substitutions for each mutant.

TPK1 alone supported the most robust growth in a *tpk* null background (15). Mutant and wild-type proteins were expressed from the endogenous *TPK2* promoter in *LEU2*-marked plasmids. Each mutation was designed to substitute a small hydrophobic residue in positions that interact with the nucleotide base to thereby create either loss of hydrogen-bonding or decreased hydrophobic interactions with the nucleotide. Each newly constructed *TPK2* mutant was then tested using standard “plasmid shuffle” assays (31) to determine whether it could provide essential PKA activity.

Transformed cells were grown selecting for both *URA3* and *LEU2* plasmids then plated in serial dilutions onto selective medium. Growth resulting from co-expression of both the wild-type and mutant plasmids was compared (Fig. 2) to that selecting against expression of wild type (5-FOA) where the only source of PKA was from the mutant *TPK2* plasmid. In most cases the *tpk2* mutants supported viability. No defects in growth were observed at either permissive (25 °C) or elevated temperatures (34 °C). One mutant, *tpk2-Y149A*, demonstrated enhanced viability at elevated temperatures. Growth appeared at least 25-fold more robust than for the wild-type control. By contrast, the *tpk2-T210V* mutant could not support viability as indicated by failure of growth on 5-FOA. The lack of viability demonstrated that this mutation caused loss of essential function.

To probe the defect caused by the *tpk2-T210V* mutation, it was coupled with additional mutations that either caused alterations in the small lobe (*tpk2-V84A*) or in the hinge region (*tpk2-M147A*), neither of which individually compromised viability. Although both residues interact with ATP within the nucleotide binding pocket, the methionine at position 147 also acts as a “molecular gate” to limit the size of molecules that bind within the pocket (34). In the case of the *tpk2-M147A,T210V* double mutant allele, viability was partially restored. This may indicate that modifications in the hinge region of the kinase help overcome defects produced by *tpk2-T210V*. In contrast, viability was not regained in *tpk* null strains expressing the *TPK2-V84A,T210V* allele. We subsequently determined that this was a dominant allele as defined below.

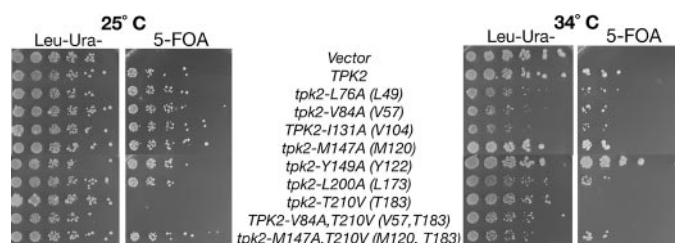


FIGURE 2. The *tpk2* pocket mutations support viability and maintain activity in a *tpk* null strain. Point mutations were constructed that caused alterations of seven residues predicted to interact with adenosine within the nucleotide binding pocket of Tpk2p. Mutant *LEU2*-marked plasmids were transformed into *tpk1* Δ *tpk3* Δ strains expressing a *URA3*-marked maintenance plasmid containing *TPK1*. Viability of the *tpk* null strain co-expressing wild-type Tpk1p and altered forms of Tpk2p was assayed on Leu–Ura– drop-out medium. To test if the *tpk2* mutant plasmids could support viability of the *tpk* null strain, growth was assessed on Leu–5-FOA medium to select against the *TPK1* plasmid. Cells were 5-fold serially diluted and grown at lower (25 °C) and elevated temperatures (34 °C). Viability was lost in the strains expressing the *tpk2-T210V* allele and the *TPK2-V84A,T210V* double mutant allele. The corresponding PKA-C α residues are in parentheses.

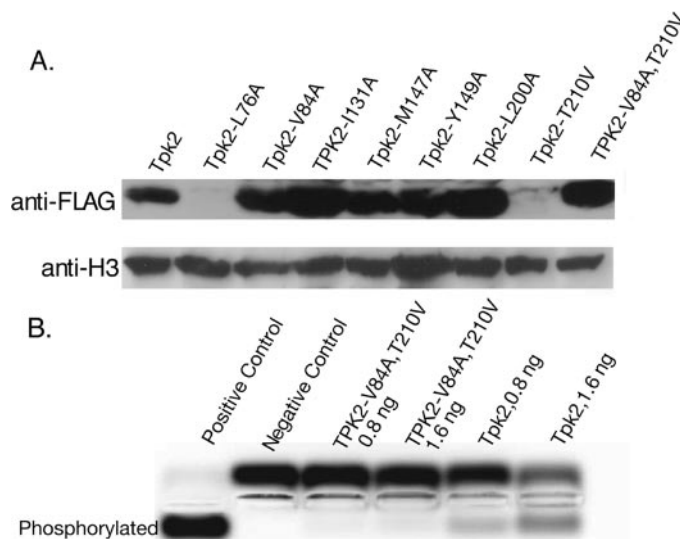


FIGURE 3. Expression and catalytic activity of altered Tpk2 proteins. A, protein expression levels of altered Tpk2 proteins were determined. Lysates from cells expressing FLAG-tagged versions of Tpk2p were separated by SDS-PAGE and probed by immunoblotting against anti-FLAG. Immunoblotting for anti-histone H3 was used as a loading control. The TPK2-V84A,T210V protein maintained high expression levels. B, enzymatic activity of the TPK2-V84A,T210V protein was compared with the wild type. Two concentrations of wild-type Tpk2p and TPK2-V84A,T210V were tested. Each reaction proceeded for 30 min before isolation of the phosphorylated peptides. Purified murine PKA-C α was used as a positive control. The negative control was performed without enzyme. The TPK2-V84A,T210V protein had loss of catalytic activity despite comparable protein expression.

Effects of Pocket Mutations on Protein Expression—To test the possibility that the lethal *tpk2-T210V* or *TPK2-V84A,T210V* mutations affected protein expression, the level of protein was evaluated for strains with the mutant plasmids (Fig. 3A). Each plasmid was constructed to encode a C-terminal FLAG tag epitope. Mutant and wild-type genes were co-expressed with unlabeled wild-type PKA to maintain comparable viability. Cultures were grown selecting for expression of both kinases, then cell lysates were prepared and analyzed by immunoblotting with anti-FLAG antiserum. Most mutant strains had levels of protein expression comparable with wild type. However, two of the altered proteins, mutants L76A and T210V, had

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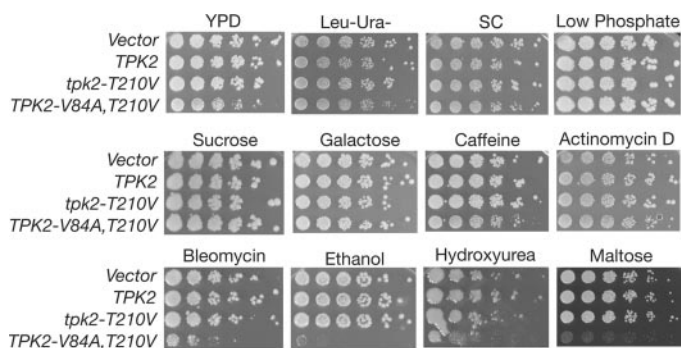


FIGURE 4. The *TPK2-V84A,T210V* allele is dominant. The plasmid-borne *tpk2-T210V* and *TPK2-V84A,T210V* alleles, which could not support viability, were co-expressed with wild-type *TPK1* expressed from a plasmid in the *tpk* null strain. Cells were plated in 5-fold serial dilutions. Dominant phenotypic defects were observed for the *TPK2-V84A,T210V* mutation for several growth conditions, including low phosphate (to test acid phosphatase induction), bleomycin (to test DNA damage repair), ethanol (to test protein stability), hydroxyurea (to probe DNA replication), and maltose (to test carbon catabolite repression). YPD, yeast extract/peptone/dextrose; SC, synthetic complete medium.

significantly less expression. Of note, the *tpk* null strain expressing either of the mutants had nearly undetectable *tpk2* protein levels yet appeared fully viable and showed only modest defects in cell signaling (Fig. 4 and supplemental Fig. S1). Thus, the L76A or T210V mutant proteins must be able to phosphorylate all essential targets despite lowered protein levels, indicating that their kinase activity may actually be quite efficient. Both sites in the wild-type protein are known to interact with adenosine within the nucleotide binding pocket, and adenosine enhances the overall stability of PKA (35). Low protein levels of these two mutants may be due in part to a significantly lowered occupancy of the nucleotide binding pocket, resulting in protein destabilization and, therefore, reduced activity. In contrast, the strain transformed with the *TPK2-V84A,T210V* allele was inviable yet had protein levels nearly equivalent to wild type. In this instance, the loss of function phenotype may be due solely to defective catalysis.

The *TPK2-V84A,T210V* Protein Has Diminished Enzymatic Activity—To address whether loss of viability in the strain expressing *TPK2-V84A,T210V* was due to loss of catalytic activity, kinase activity was qualitatively assayed (Fig. 3B). Wild-type and mutant proteins were partially purified by FLAG affinity and tested for their capacity to phosphorylate a peptide with the PKA substrate consensus sequence (LRRASLG) (36). Phosphorylation of this model peptide was monitored over 30 min at 2 protein concentrations. The phosphorylated peptides were then separated from non-phosphorylated peptides by electrophoresis. When compared with wild-type activity, *TPK2-V84A,T210V* was severely impaired and catalyzed little to no phosphorylation of the peptide. This demonstrated that the double point mutations at sites V84A and T210V caused a significant loss of enzymatic activity.

The *TPK2-V84A,T210V* Protein Interfered with Normal Function of PKA—Because the *tpk* null strain expressing either the *tpk2-T210V* allele or the *TPK2-V84A,T210V* allele was non-viable, we asked whether these mutations had additional *in vivo* consequences. The mutant alleles were co-expressed with wild-type *TPK1* to maintain viability and assayed in a broad survey of growth conditions (32). There appeared to be no effects for

many of the physiological challenges, but the *TPK2-V84A,T210V* mutation was dominant under several conditions (Fig. 4). Viability was moderately to severely impaired under a variety of circumstances, including growth when bleomycin, ethanol, or hydroxyurea were added to nutrient-rich medium or when maltose was the only carbon source. These phenotypes suggest potential interference with acid phosphatase induction, DNA damage repair, protein stability, DNA replication, and carbon catabolite repression (32). Such dominance indicates that although the *TPK2-V84A,T210V* protein cannot phosphorylate essential substrates needed for survival, it appears to interfere with the wild-type Tpk function in several different signaling pathways.

Restoring Function to the *TPK2-V84A,T210V* Mutant Through Intragenic Suppression—Modulation of kinase activity can be coordinated through multiple, distal regulatory sites (2), but identification of mediators of communication between these sites has proven difficult. We reasoned that an unbiased genetic approach might prove productive. Because *TPK2-V84A,T210V* had the greatest catalytic defect both *in vivo* and *in vitro*, this allele was used as the starting point to identify suppressor mutations that could restore catalytic activity to Tpk2p. Libraries were constructed after random mutagenesis of a *TPK2* template bearing the double point mutant. The libraries were transformed and then tested using the plasmid shuffling approach described above and diagrammed in Fig. 5A.

Library mutation rates varied from 1 to 20 mutations per 1000 base pairs. Approximately 25,000 transformants were screened for ability to grow without the covering *TPK1*-bearing plasmid (Fig. 5). Plasmids were recovered from these suppressors and re-transformed into the *tpk* null strain to establish that the ability to grow was reproducibly bestowed by the plasmid.

Multiple suppressor plasmids were isolated and sequenced for identification of their newly acquired mutations (Table 2 and Fig. 6). Several mutations were found within the proximity of the nucleotide binding pocket. Because the original mutations were introduced at sites that directly interact with the adenosine base, it was perhaps not surprising that many suppressor mutations mapped to areas that could directly assist in enhancing the nucleotide binding event. No suppressors were identified that directly reverted either of the original mutations at sites Val-84 and Thr-210.

Other suppressors represented mutations affecting the C-terminal region within the conserved kinase core. Although distal to the nucleotide binding pocket, this region assists in kinase activity by serving as a substrate binding site as well as by positioning the activation loop for catalysis (27). Many suppressor mutations in this region mapped to solvent-exposed sites of the large lobe. This may be indicative of altered substrate interactions in which kinase activity may be restored through enhanced protein interactions rather than directly through improved catalytic activity.

One suppressor mutation that was isolated several times was within the sequence encoding the non-conserved N-terminal tail of Tpk2p at site Pro-55. This particular residue is positioned after the polyglutamine-rich stretch in the N terminus of Tpk2p that distinguishes it from most other members of the kinase superfamily. It is possible that loss of structural rigidity resulting

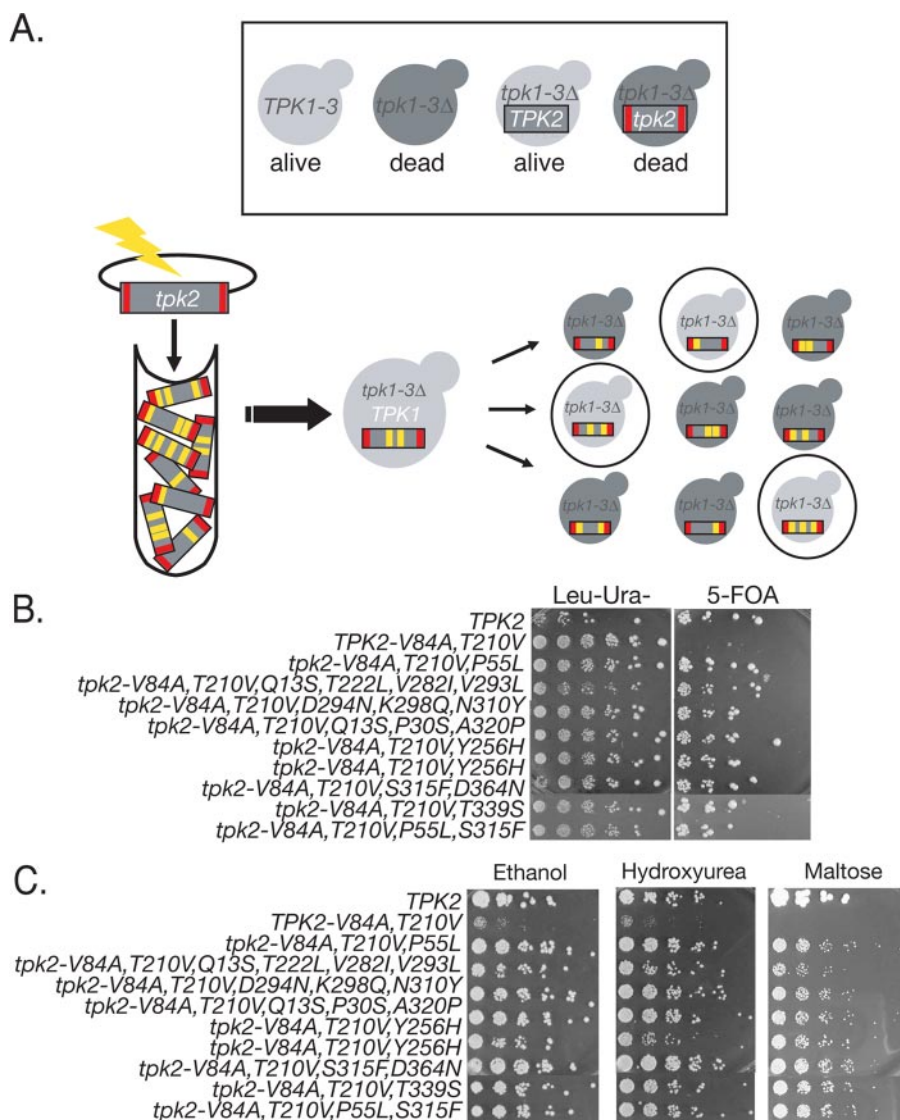


FIGURE 5. Identifying suppressors of the *TPK2-V84A,T210V* mutant. A, identifying suppressors of the double mutant. The boxed diagram summarizes relevant genotypes and phenotypes of the PKA mutants in yeast. The cell at the far right depicts the observation that the *TPK2-V84A,T210V* mutant cannot survive without another source of PKA. To identify suppressors, the *TPK2-V84A,T210V* plasmid was used to create further mutagenized libraries. These were transformed into the *tpk* null mutant and then assayed for ability to restore *TPK2* function. Cells that can live with no other source of PKA bear candidate intragenic suppressors. B, because a plasmid bearing the *TPK2-V84A,T210V* allele could not support viability of the *tpk* null strain, the extent of rescue was measured for plasmids bearing a representative sample of suppressors identified in this study. Transformants were assayed as in Fig. 2. All suppressors (Table 2) had comparable results. C, the dominance shown in strains expressing the *TPK2-V84A,T210V* allele was not seen in strains expressing the suppressor alleles. Cultures were 5-fold serially diluted, plated on the indicated phenotyping plates, and grown for 6 days at 30 °C. All suppressors had comparable results.

from the P55L mutation may partially compensate to restore kinase activity.

The suppressors were also tested to determine the extent to which they independently supported viability in the absence of wild-type PKA (Fig. 5B). The *tpk* null strain bearing the *TPK1* plasmid was transformed with plasmids containing the suppressor alleles as well as the control wild-type *TPK1* allele. Transformants were again tested for growth by 5-fold serial dilution on medium that selected for maintenance of both plasmids or that selected against the *TPK1*-containing plasmid. All of the suppressor alleles tested fully supported growth in the

background of the triple *tpk* null strain, with no other source of PKA.

Physiological Defects of the *TPK2-V84A,T210V* Double Mutant Were Rescued by the Suppressors—Because the *TPK2-V84A,T210V* mutant dominantly interfered in several physiological pathways (Fig. 4), the suppressor plasmids that restored essential *TPK2* functions were tested to determine whether the dominant defects in signaling were also relieved. Strains expressing suppressor mutants were grown on medium containing ethanol, hydroxyurea, or maltose to test for dominance (Fig. 5C). The suppressor mutants grew robustly under these conditions, demonstrating both that normal PKA activity was fully restored and that interference with wild-type function was also relieved. Of note, no suppressors were identified that only restored either the dominant negative phenotype with wild-type PKA or defects in PKA signaling. Because suppressors were initially identified solely by their ability to restore viability in the *tpk* null strain, the screen may favor recovery of loss of dominant interference. Additionally, it may be anticipated that viability will be enhanced in strains with fully active cell signaling from Tpk2p, thereby these strains may be more readily identified from the screen as it was originally designed.

DISCUSSION

Kinases are critical regulators of intracellular signaling, the functions of which can range from essential activities to fine-tuning physiological responses to the environment (1). Protein kinase A is a particularly well studied representative of the

kinase family that regulates multiple signaling pathways and is essential for viability. This kinase also defines a structural model for the catalytic core that is conserved throughout the superfamily (24). To probe how kinases differentially regulate their activity, the PKA homolog Tpk2p was used as to investigate how both conserved and non-conserved sequences can influence its activity *in vivo*.

Functional Roles of Nucleotide Binding Residues Are Not Conserved—The studies reported here provide *in vivo* analysis of mutations predicted to compromise nucleotide binding. Mutation of sequences encoding residues affecting nucleotide

TABLE 2

Suppressor *TPK2* mutant plasmids identified in this study

Plasmid	Description ^a	Corresponding PKA-Cα residues
pLP2043	<i>pLP2042</i> (P55L (CCC→CTC); 261 (GGT→GGG); S315F (TCC→TTC))	(Pro-55), Val-288
pLP2044	<i>pLP2042</i> (P55L (CCC→CTC); S315F (TCC→TTC))	(Pro-55), Val-288
pLP2045	<i>pLP2042</i> (M109T (ATG→ACG); K171N (AAA→AAT); 261 (GGT→GGG))	Leu-82, Arg-144
pLP2046	<i>pLP2042</i> (E13S (CAA→TCA); 155 (CTT→CTG); T222I (ACA→ATA); V282I (GTA→ATA); V293L (GTG→CTG))	(Gln-13), Thr-195, Val-255, Lys-266
pLP2048	<i>pLP2042</i> (94 (CGG→CGA); D294N (GAT→AAT); K298E (AAA→CAA); 309 (GGT→GGG); N310Y (AAT→TAT))	Asp-267, Asn-271, Asn-283
pLP2049	<i>pLP2042</i> (E13S (CAA→TCA); P30S (CCG→TCG); 174 (GCT→GCC); 270 (CCA→CCC); 309 (GGT→GGG); A320P (GCT→CCT))	(Gln-13), (Pro-30), Asn-293
pLP2050	<i>pLP2042</i> (Y256H (TAC→CAC))	Tyr-229
pLP2052	<i>pLP2042</i> (306 (GAA→GAG); S315F (TCC→TTC); 324 (TTT→TTC); D364N (GAT→AAT))	Val-288, Val-337
pLP2059	<i>pLP2042</i> (282 (GTA→GTG); 324 (TTT→TTC); T339S (ACT→AGT))	Ala-312
pLP2060	<i>pLP2042</i> (P55L (CCC→CTC); S315F (TCC→TTC))	(Pro-55), Val-288
pLP2061	<i>pLP2042</i> (P55L (CCC→CTC); S315F (TCC→TTC))	(Pro-55), Val-288
pLP2062	<i>pLP2042</i> (P55L (CCC→CTC); S315F (TCC→TTC))	(Pro-55), Val-288
pLP2063	<i>pLP2042</i> (P55L (CCC→CTC); 83 (AGG→AGA); 211 (GAT→GAC); S315F (TCC→TTC))	(Pro-55), Val-288
pLP2064	<i>pLP2042</i> (P55L (CCC→CTC); S315F (TCC→TTC))	(Pro-55), Val-288
pLP2065	<i>pLP2042</i> (51 (ACG→ACA); Y256H (TAC→CAC))	Tyr-229
pLP2066	<i>pLP2042</i> (G63S (GGC→ACG); L161E (AAG→CAG); S250Y (TCT→TAT))	Asn-36, Arg-134, Ala-223
pLP2067	<i>pLP2042</i> (R120S (CGT→AGT); E217D (GAG→GAT))	Arg-93, Arg-190
pLP2068	<i>pLP2042</i> (D267H (GAT→CAT))	Ala-240
pLP2069	<i>pLP2042</i> (30 (CCG→CCA); 108 (AAG→AAA); K208N (AAA→AAT))	Gln-181
pLP2070	<i>pLP2042</i> (30 (CCG→CCA); 108 (AAG→AAA); K208N (AAA→AAT); H289R (CAT→CGT))	Gln-181, Ser-262

^a Note that the description of each plasmid specifies both nucleotide and amino acid substitutions for each mutant suppressor identified. The equivalent residues for suppressor mutations in PKA-Cα are listed. *TPK2* mutations that map outside the conserved PKA-Cα sequence are listed in parentheses.

binding and expression of the corresponding mutant alleles in a *tpk* null strain allowed examination of the significance of each residue within its cellular context. Because of the insoluble nature of recombinantly expressed Tpk2p, it has remained largely uncharacterized biochemically. However, considerable work has been performed on recombinantly expressed Tpk2p homologs to examine the catalytic effects of alteration of these sites. Despite extensive *in vitro* characterization of Tpk2p mutant homologs before this study, little was known about their biological effects (34, 37).

For example, alterations at the molecular gate at position Met-147 can cause loss of activity *in vitro* that can be partially compensated by an additional change at the equivalent T210A/T210G position in mouse PKA isoforms (34, 38). However, in our studies no *in vivo* effect was associated with the introduction of the *tpk2-M147A/M147G* mutations. Instead, when mutations were introduced into sequences encoding the same position of the yeast isoform Tpk2p, the opposite results were found, defining a clear requirement for Thr-210. These data, therefore, establish that the Met-147 and Thr-210 positions are not functionally redundant for Tpk2p and possibly for other PKA isoforms. Despite significant identity within the nucleotide binding pocket, the importance of each individual residue may differ for modulation of ATP binding. Seemingly subtle differences may assist in refining kinase regulation by introducing minor alterations in the nucleotide binding pocket.

Multiple positions that were predicted to perform major roles in promoting nucleotide binding did not cause significant functional defects when altered. In contrast, positions that structurally appeared to provide seemingly minor hydrophobic interactions had dire consequences *in vivo* when altered.

An unexpected result was the interference with wild-type activity by the *TPK2-I131A* allele at elevated temperatures. This mutation lies near the area encoding the dynamic hinge region of the kinase core between the small and large lobes that allows the kinase to open and close during nucleotide binding. Muta-

tions in this region have not been found to cause significant defects in catalytic activity but can affect binding by nucleotides and nucleotide analogs (34). Although the TPK2-I131A protein is clearly capable of utilizing ATP, it interferes with the function of wild-type Tpk2p. Tpk2p has been predicted to interact directly with both Tpk1p and Tpk3p (39, 40); thus, TPK2-I131A is likely to sequester or prevent wild-type activity, and this may be mediated by altered lobe dynamics stemming from this mutation.

The *TPK2-V84A,T210V* mutant allele also interfered with wild-type activity under diverse physiological challenges. Although the TPK2-V84A,T210V protein is catalytically inactive, it is indeed expressed. It is possible that this altered protein may affect wild-type activity through direct interactions or that its regulation by the PKA regulatory subunit Bcy1p may be altered. Bcy1p exists as a homodimer that is capable of binding two catalytic subunits of PKA when cAMP concentrations are low, thereby keeping the catalytic subunits inactive. Perhaps altered affinity for Bcy1p with the TPK2-V84A,T210V protein leads to lower occupancy in the regulatory complex, resulting in an increased sequestered state of the wild-type enzyme.

Areas Distal to the Nucleotide Binding Pocket Are Significant in Modulating Activity—Starting from mutations in *TPK2* that caused defective kinase activity, intragenic suppressors were identified that restored kinase activity. The initial mutations were introduced within the highly conserved kinase core. Several suppressor mutations were identified in proximity to the nucleotide binding pocket and are likely to play direct roles in restoring nucleotide binding in the *TPK2-V84A,T210V* background. Because the initial mutations were predicted to disrupt the nucleotide binding event, it was likely that suppressor mutations might restore this aspect of kinase activity. Surprisingly, many suppressors were found in regions of the kinase that are distant to this site. This finding has been observed for other proteins, including kinases, where conserved networks appear to mediate allosteric regulation of the kinase core (41, 42). Sev-

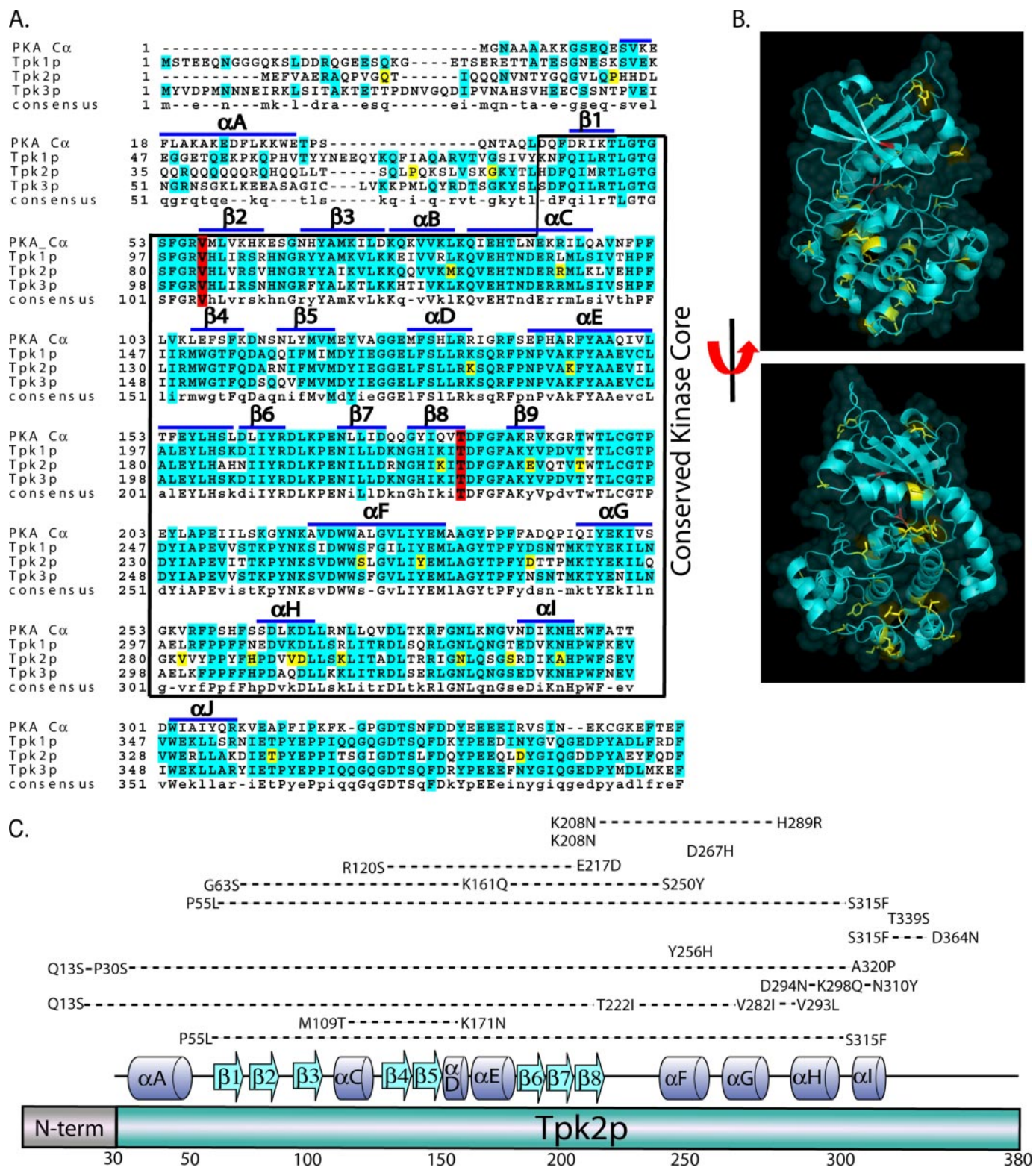


FIGURE 6. Suppressor mutations of Tpk2p. A, sequence alignment of Tpk2p with *S. cerevisiae* and *Mus musculus* isoforms. Site-directed mutations at positions Val-84 and Thr-210 are highlighted in red. Suppressor mutations are in yellow, and conserved residues are in cyan. Many mutations are clustered at sites encoding residues within the C terminus of the kinase core. Multiple suppressors were identified to encode a change at position P55. B, sites of suppressor mutations within the conserved kinase core are structurally represented with a 180-degree rotation. Suppressor mutations are in yellow. Initial, site-directed mutations within the nucleotide binding pocket are in red. Sites of suppressor mutations are represented at the corresponding sites of murine PKA-Cα using Protein Data Bank code 1ATP and modeled using PyMOL. C, suppressor mutations are represented over a secondary structure model of PKA. Multiple mutations occurring from a single suppressor are connected by dashes. The non-conserved N-terminal extension is highlighted in gray.

Distinct Regions of Tpk2p Mediate Activity

eral mapped to regions encoding the non-conserved N-terminal region of Tpk2p, but the majority of suppressors mapped to the large lobe of the kinase domain. The large lobe performs several critical roles including substrate docking and positioning of the activation loop (43). The mutations identified underscore the functional significance of this area for Tpk2p catalysis. Additionally, the suppressors demonstrate that restoration of kinase function may occur in a manner that will prove unique for each individual kinase and may be mediated via both conserved and non-conserved features for enzyme regulation.

An example is the suppressor *tpk2-R120S,E217D*. These mutations correspond to sites Arg-93 and Glu-190 in PKA that have been shown to be key interactors with the non-conserved A-helix (44). Arg-93 is near the end of the C-helix of the small lobe, whereas Glu-190 is a solvent-exposed residue that sits at the end of β -strand 9 of the large lobe. These sites form a socket to promote anchoring of Trp-30 from the A-helix (supplemental Fig. S2A). This socket is formed at the hinge region between the large and small lobes. These suppressor mutations may, therefore, influence the stability of A-helix interactions with the kinase domain, thereby indirectly influencing hinge dynamics and, therefore, kinase activity.

Residue Arg-93 is also positioned adjacent to Lys-92 in the C-helix. This is a conserved residue in the kinase family that helps form a docking site for regulatory proteins thereby allowing the C-helix to assume the active conformation (45). Disease-causing mutations have been frequently found at this site among multiple members of the kinase family (46). The mutation isolated at Arg-120 (Arg-93) may reflect an extension of this regulatory site where interactions with key regulatory effector proteins may be altered.

Another suppressor, *tpk2-D294N,K298Q,N310Y* (PKA-C α sites Asp-267, Asn-271, and Asn-273, respectively), contains mutations that are all solvent-exposed and lie at the base of the large lobe (supplemental Fig. S2B). These residues reside at a region that is critical for interactions with the RII regulatory subunit but not the RI regulatory subunit of PKA.⁵ The PKA subunit in yeast, Bcy1, is homologous to the RII subunit. These suppressor mutations may help loosen the otherwise stringent regulation by Bcy1. Although initial co-immunoprecipitation experiments of Bcy1 and PKA have not demonstrated a significant change in interactions for several suppressors,⁶ subtle differences may be sufficient for causing a larger phenotypic change within the cellular context. Further biochemical studies investigating the interactions of Tpk proteins with Bcy1 should provide insight into the regulation of PKA activity.

Another site of suppressor clustering was among N-terminal residues. This region flanks the core but diverges from other enzymes of the kinase superfamily. The identification of suppressors in this region demonstrates that it can influence kinase activity and may additionally explain the specificity of unique roles of Tpk2p in signaling pathways including those regulating pseudohyphal growth, respiratory growth, and flocculation (18, 19, 21, 47, 48). Although structural data are not yet available for

this isoform-specific region, it may prove of significance in understanding how this region modulates activity.

Because suppressors were acquired from a mutant kinase background, it will be interesting in future studies to see the effects of individual mutations within the context of the wild-type protein. Because several residues that are known to have direct involvement in catalytic activity caused no phenotypic effects when mutated, it may be anticipated that some of the individual suppressor mutations will be inconsequential when introduced into a wild-type protein. However, it is possible that some suppressor mutations may indeed cause dominant effects. It will be of interest to identify such residues or to look for them directly to gain a broader perspective on the regulatory mechanisms of PKA in yeast.

Eukaryotes have multiple functional PKA isoforms, perhaps indicating both harmonized and antagonistic biological functions for cell signaling. Although much progress has been made to identify unique substrates for each isoform, it has remained difficult to deconvolute their unique roles (49). The results from this analysis demonstrate that both conserved and non-conserved features of PKA perform significant roles in governing kinase activity. Understanding how PKA isoforms have evolved both to complement and distinguish their activities from one another may provide critical insight into how PKA regulates signaling responses.

Genetic Screening as a Tool for Identifying Intramolecular Elements of Kinase Regulation—Modulation of activity by non-conserved regions of a kinase provides unique platforms for kinases to achieve distinction and specificity from one another (supplemental Fig. S3). Kinases have been implicated in a variety of diseases, including non-small-cell lung cancer, prostate cancer, leukemia, neuronal developmental disorders, and multiple sclerosis (50–53). For many diseases, several kinase inhibitors have been developed that target the highly conserved nucleotide binding pocket and have had initial therapeutic successes. However, several inhibitors including those currently used therapeutically such as gefitinib, imatinib, and erlotinib affect a broad range of kinases (54), and clinical resistance to several kinase inhibitors has been observed via additional mutations within the catalytic domain (55–57). The ability to identify and target regions that are kinase-specific yet distal to the nucleotide binding pocket may assist in improving the specificity and utility of therapeutic kinase modulators.

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SUPPLEMENTARY MATERIAL

To determine if strains expressing viable pocket alterations had any signaling defects, plasmids containing the various viable pocket mutant alleles of *TPK2* were transformed into the *tpk* null strain expressing the *TPK1-URA3* plasmid, grown on 5-FOA to cure the strain of the *TPK1-URA3* plasmid, and tested for growth under a number of conditions. Cells were plated in 5-fold serial dilutions and incubated for 6 days at 30° C on medium containing compounds that highlight defects under a variety of distinct signal transduction pathways (Fig. S1). For almost all conditions tested, the strains bearing the *tpk2* mutant plasmids demonstrated no growth defects. Modest defects were seen for plasmids bearing *tpk2-L76A* and *tpk2-M147A*, *T210V* when grown with galactose as the sole carbon source or with the DNA damaging agent bleomycin, suggesting partial defects in transcriptional activation and DNA damage repair caused by ionizing or radiomimetic agents, respectively (30). The most significant phenotypic defect was observed when grown in medium containing caffeine, suggesting potential defects in components of the MAP kinase signaling pathway (30). The strains that demonstrated this defect bore plasmids containing *tpk2-L76A*, *tpk2-Y149A*, and *tpk2-L200A* alleles, as well as the *tpk2-M147A*, *T210V* double mutant allele.

FIGURE S1 Phenotypic analysis of nucleotide binding pocket mutants that supported viability. Mutant plasmids that did not confer viability defects when expressed as the sole source of PKA in the *tpk* null strain were further tested to determine whether any signaling pathways were affected. Transformants were 5-fold serially diluted and grown on a variety of phenotype media for 6 days at 30° C. Slight defects were seen for strains grown on bleomycin and galactose. Strong phenotypes were observed for several strains grown on caffeine, an indicator of effects on the MAP kinase signaling pathway.

FIGURE S2 Selected suppressor mutations. *A*, Critical interactions between the A-helix and the large and small lobes are demonstrated by the *tpk2-V84A,T210V,R130S,E217D* suppressor. Sites R130 and E217 correspond to sites R93 and R190 in PKA-C α . These sites derive from the small and large lobes and interact with W30 of the A-helix. These interactions are formed at the hinge region between the kinase lobes and promotes stability. *B*, Critical interactions between the kinase and regulatory domains. The suppressor *tpk2-V84A,T210V,D294N,K298Q,N310Y* acquired mutations that reside at the base of the small lobe and are solvent-exposed (corresponding PKA-C α sites D267, N271, and N283). This area of the kinase has been shown to be critical for interactions with the RII regulatory subunit of PKA.

FIGURE S3 Mutations acquired within the N-terminal extension of Tpk2p. Sequence alignment of the non-conserved N-terminal extensions of the TPK proteins as well as PKA. Suppressors were identified by viability screening and mutations were identified by DNA sequencing. Suppressor mutations acquired within this region are highlighted in red. Many mutations lie within the unique poly-glutamine region of Tpk2p as well as at site P55.

Figure S1

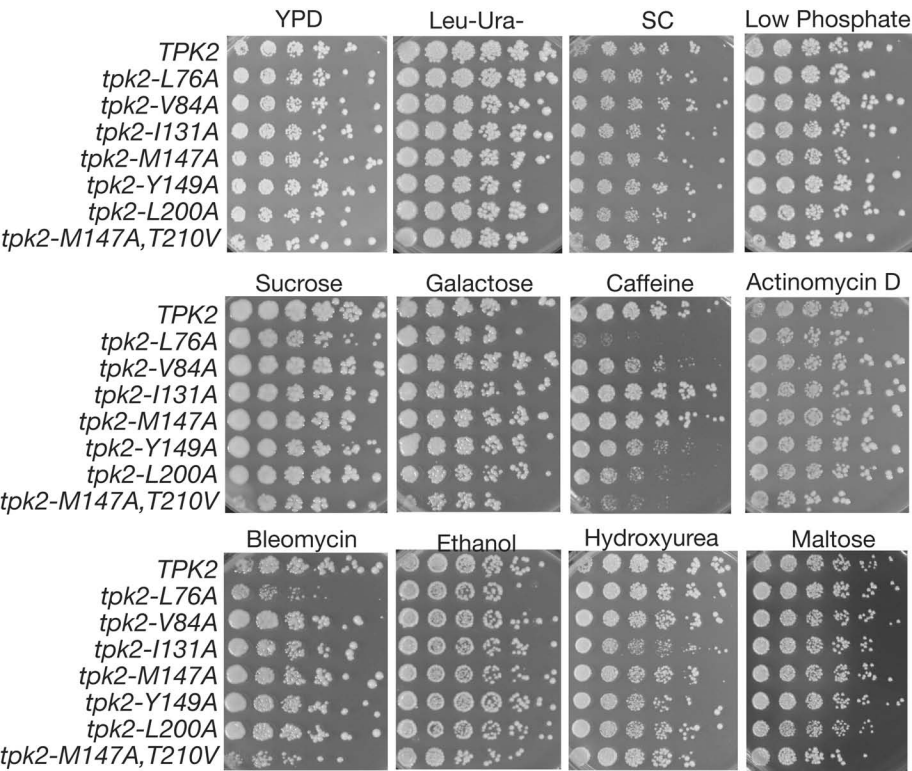
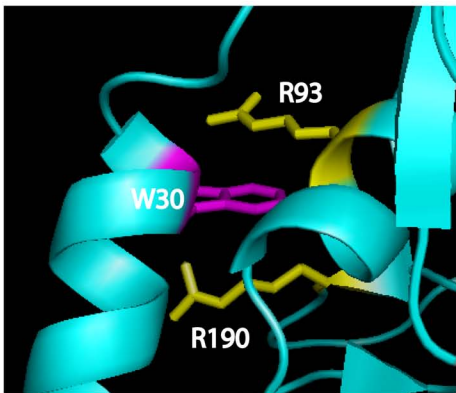


Figure S2

A.



B.

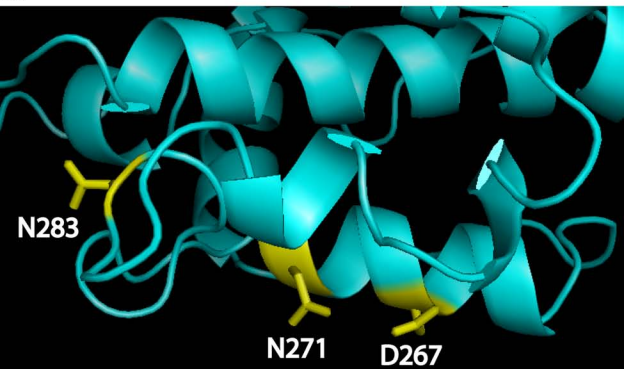


Figure S3

A.

PKA_C α	1	-----MGNAAAAKKGSEQESVKE	
Tpk1p	1	MSTEEQNGGGQKSLDDRQGEESQKG---ETSERETTATESGNESKSVEK	
Tpk2p	1	-----MEFVAERAQPVGQT-----IQQQNVNTYGGVLPQHDL	
Tpk3p	1	MYVDPMNNNEIRKLSITAKTETTPDNVGQDIPVNAHSVHEECSSNTPVEI	
consensus	1	m--e--n--mk-l-dra--esq-----ei-mqn-ta-e-gseq-svel	

PKA_C α	18	FLAKAKEDFLKKWETPS-----QNTAQL	40
Tpk1p	47	EGGETQEKPKQPHVTYYNEEQYKQFIAQARVTVGSIVY	83
Tpk2p	35	QQRQQQQRHQQLLT-----SQLQKSLVSKSKYTL	67
Tpk3p	51	NGRNSGKLKEEASAGIC---LVKKPMLQYRDTSGKYSL	84
consensus	51	qgrqtqe--kq---tls-----kq-i-q-rvt-gkysl	

B.

G63S
P55L
Q45R
Q40L/S
Q39H/Y
Q38H
H31Q
Q25H/P

Tpk2p

**Identification of Functionally Distinct Regions That Mediate Biological Activity of
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Eileen J. Kennedy, Gourisankar Ghosh and Lorraine Pillus

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