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Drosophila protein kinase CK2 is rendered temperature-sensitive by mutations of highly conserved residues flanking the activation segment

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Abstract

CK2 is a Ser/Thr protein kinase essential for animal development. Although null alleles for CK2 are available in the mouse and *Drosophila* models, they are lethal when homozygous, thus necessitating conditional alleles for analysis of its developmental roles. We describe the isolation of temperature-sensitive (ts) alleles of *Drosophila* CK2 α (dCK2 α). These alleles efficiently rescue lethality of yeast lacking endogenous CK2 at 29°C, but this ability is lost at higher temperatures in an allele-specific manner. These ts-variants exhibit properties akin to the wild type protein, and interact robustly with dCK2 β . Modeling of these ts-variants using the crystal structure of human CK2 α indicates that the affected residues are in close proximity to the active site. We find that substitution of Asp²¹² elicits potent ts-behavior, an important finding because this residue contributes to stability of the activation segment and is invariant in other Ser/Thr protein kinases.

Keywords

CK2; protein kinase; temperature-sensitive; *Drosophila*

Introduction

CK2 is a Ser/Thr protein kinase that is ubiquitous and highly conserved (reviewed in [1,2]. This enzyme has been purified from diverse organisms such as yeast, insects and mammals, and is composed of catalytic α subunits and regulatory β subunits. The CK2 holoenzyme is formed by the association of two catalytic α subunits with a core-dimer of the regulatory β subunits [3-5]. In most organisms, there are two distinct CK2 α subunits, α and α' , which are encoded by distinct genes. This, however, is not the case in the fruit fly *Drosophila melanogaster* and the fission yeast *Schizosaccharomyces pombe*, which harbor a single CK2 α gene [6,7]. In contrast, budding yeast contain two catalytic subunits, α and α' , which are encoded by the *CKA1* and *CKA2* genes, respectively. It is important to, however, note that the yeast α/α' subunits do not functionally correspond to metazoan α/α' subunits; the nomenclature in yeast reflects the electrophoretic migration of these subunits and the order in which the corresponding genes were isolated and sequenced [8-10]. In contrast, most organisms contain a single isoform of CK2 β . However, multiple isoforms of CK2 β , which are encoded by distinct genes, have been reported in *Saccharomyces cerevisiae* and *Drosophila* [10-14]. Despite these differences in subunit composition, CK2 activity is messenger-independent, and this enzyme preferentially phosphorylates Ser/Thr residues that meet the consensus S/T-E/D-x-E/D [15,

16]. Given the unique micro-acidic determinant for phosphorylation, computational approaches have been employed to identify potential targets of this enzyme [17,18]. The preponderance of potential CK2 targets, which include proteins involved with transcription, translation, cell cycle, and cell signaling, are being confirmed by proteomic approaches [19], suggest that this enzyme performs higher order functions (reviewed in [20]).

Genetic studies in yeast were the first to demonstrate that CK2 is an essential enzyme. In budding yeast, deletion of both the *CKA1* plus *CKA2* genes is lethal, and cells depleted of CK2 exhibit a pseudomycelial morphology, reminiscent of *cdc* mutations [9]. It was subsequently shown (using *CKA2*-tsalleles) that loss of CK2 activity elicited cell cycle arrest at the G1/S and G2/M transitions [21], thus establishing this enzyme as a regulator of cell proliferation, a role also evidenced for mammalian CK2 [22], and a lymphoproliferative disorder called Theileriosis [23]. Aside from its roles in functions that are cell-autonomous, it is becoming increasingly evident that CK2 activity is important for animal development. For example, CK2 regulates early embryonic development via the phosphorylation of proteins such as Antennapedia, Engrailed, Odd-skipped and Ultrabithorax [24-27]. More recently, the roles of this enzyme have been described in wingless/wnt signaling, and for Notch signaling during neurogenesis [28-31].

To better define the developmental roles of CK2, mutants are necessary. However, null alleles of CK2 elicit lethality early in mouse development due to cell-autonomous defects [32-34]. In this regard, *Drosophila* is a widely studied and an appropriate model for studying human disease and mammalian development (reviewed in [35]). A null allele of *Drosophila CK2α*, named *Timekeeper* (*Tik*), was identified in a screen for modifiers of the circadian clock [36]. Animals heterozygous for *Tik* display a pronouncedly lengthened behavioral rhythm, suggesting that this allele acts in a dominant manner [37]. Molecular analysis demonstrated that the *Tik* allele harbors two missense mutations, which replace Met¹⁶¹ with Lys and Glu¹⁶⁵ with Asp. Of these two residues, Met¹⁶¹ is invariant in all CK2α subunits and lies within the ATP binding pocket [38]. Accordingly, its substitution with Lys impedes ATP-binding and inactivates kinase activity, a finding confirmed for a *Timekeeper* variant of human CK2α [39]. Because of this inactivity, *Tik* homozygotes are larval lethal. However, *Tik*/+ flies do not display any overt developmental abnormalities, and thus embryogenesis, segmentation, and neurogenesis proceed normally [28,36,40]. As a result, RNAi approaches have been used to assess its roles in neurogenesis [28], but the perdurance of preformed enzyme has precluded analysis of early embryonic functions or in other developmental programs. A similar situation exists for *Drosophila CK2β*, for which hypomorphic and null alleles have been identified. The hypomorphic allele *andante* (replaces Met¹⁶⁶Ile) also affects the circadian clock, but animals homozygous for the *andante* allele do not display any overt developmental defects [41]. An insertion of a transposable (*P*) element in *CK2β* was subsequently identified, and named *CK2β^{mbuP1}* because these flies displayed a ‘mushroom body undersized (*mbu*)’ phenotype due to hypo-proliferation of cells that comprise this structure. Imprecise excision of this P-element resulted in deletion of the *CK2β* gene (called *CK2β^{mbuΔA26}*), which was embryonic lethal when homozygous [42], as in also the case for mouse *CK2β* [32]. However, *CK2β^{mbuΔA26}*/+ flies do not display any overt developmental defects. Together, these studies suggest that the circadian clock is highly sensitive to CK2 dosage, whereas other developmental programs are perhaps more buffered.

In order to bypass the lethal effects associated with a complete loss of CK2 functions, two complementary approaches have been employed. These involve the identification of substrates of this enzyme, and the role of phosphorylation is then confirmed by analyses of phenotypes following expression of variants that are non-phosphorylatable or mimic the constitutively phosphorylated substrate. This approach has been successfully employed to assess CK2 functions in embryogenesis and neurogenesis [24-26,29]. Alternatively, RNAi-based

approaches have also been used [28]. However, these approaches are not applicable to all developmental programs, and thus necessitate conditional (ts) alleles.

We describe here the isolation and characterization of ts-variants of dCK2 α using a yeast complementation system. We show that these variants exhibit significant similarity to the biological properties of wild type CK2 α , but are rendered non-functional at higher temperatures in an allele-specific manner. Importantly, we show that replacement of Asp²¹² with Asn elicits potent ts-behavior. This finding is of general relevance, because Asp²¹² is invariant in the Ser/Thr protein kinase family, and plays a critical role in stabilizing the activation segment.

Materials and Methods

Construction of Ts alleles

The complete open reading frame of wild-type dCK2 α encompassing the region from Met¹-Gln³³⁶ (Genbank accession number, M16534, [6]) was amplified by PCR to introduce BamH1 and Xho1 sites at the 5' and 3' ends, respectively. The PCR product was subcloned into pBSII (KS+) (Stratagene) and completely sequenced to confirm the absence of any PCR errors. The dCK2 α cassette was then subjected to PCR-based site-directed mutagenesis to generate the putative ts-variants, dCK2 α -A¹⁷⁷T, dCK2 α -A¹⁷⁷V, dCK2 α -D²¹²N, and dCK2 α -G⁸⁹D. The selection of these sites was based on extant ts-alleles of yeast *CKA2* (see Fig. 1A). Sequences of primers used for mutagenesis are available upon request. The resulting PCR products were subcloned into pBSII(KS+) and verified by sequencing.

Rescue of yeast harboring null alleles of yeast *CKA1* plus *CKA2*

Constructs encoding The WT/mutant dCK2 α variants were subcloned into the BamH1 and Xho1 sites of the vector pESC-*LEU* (Stratagene). This vector utilizes a divergent *GALI/10* promoter that enables inducible expression of heterologous proteins in yeast. Similarly, dCK2 α -WT was cloned into the BamH1 and Xho1 sites of the vector pESC-*URA* (Stratagene). In both cases (pESC-*LEU* or pESC-*URA*), expression of dCK2 α -WT or its site-specific variants is under control of the *GALI* promoter.

The yeast strain RPG41-1a was utilized as a host for these studies [9]. This strain is deleted for both the *CKA1* and *CKA2* genes and is rescued by a single copy *CEN/ARS-URA3*-marked plasmid harboring a wild-type copy of the *CKA2* gene. Strain RPG41-1a was transformed with the pESC-*LEU* constructs containing the WT or putative ts-alleles of dCK2 α using lithium acetate. Transformants harboring both plasmids were isolated on synthetic minimal dextrose (SM-Dex) media containing adenine and lysine, and the resulting strains were named YKP1 through YKP5 (Table 1). These strains were grown non-selectively in rich galactose media (YPG) to drive expression of dCK2 α constructs. Finally, ~300 cells were plated on synthetic minimal galactose (SM-Gal) media supplemented with adenine, lysine, uracil and 0.75 mg/ml 5-Fluoroorotic acid (5 FOA, Zymogen). 5FOA is toxic to cells containing a wild type *URA3* gene. Survivors of this selection have dropped the (*URA3*-marked) *CKA2*-encoding plasmid, and their inviability is thus rescued by dCK2 α -constructs contained in the vector pESC-*LEU*. The resulting strains, named YKP6-10, were reconfirmed for uracil auxotrophy. Growth was assessed by spotting ~250 cells of each strain followed by incubation at temperatures ranging from 25°C to 37°C.

Yeast phenotypic analyses

Strains were inoculated in YPG at a starting density of 2.5×10^5 cells/ml. Cultures were grown at the permissive temperature (29°C) with shaking at 300 RPM. Aliquots were removed and fixed with formaldehyde at a final concentration of 3.7%. Cell numbers were determined using a hemocytometer, and the mean generation times were determined from semi-log plots.

Percent flocculation was determined as described [14]. Briefly, yeast strains were grown to mid-log phase at 29°C with shaking at 300 RPM. Cultures were allowed to stand at room temperature for 5 min, and free cells in the supernatant were determined based on absorbance at 600 nm (A_{600}). EDTA was added to the remaining culture at a final concentration of 20 mM to disperse the flocculated cells and to estimate total cells by A_{600} . Percent flocculation was calculated as $100 \times [(A_{600} \text{ total} - A_{600} \text{ free}) / A_{600} \text{ total}]$.

Terminal phenotypes were determined for strains rescued by dCK2 α constructs. Following growth to mid log phase at 29°C, cultures were transferred to the non-permissive temperature (37°C). Samples were collected prior to, and 22 hr after the shift to non-permissive temperature, fixed with formaldehyde at a final concentration of 3.7% and photographed under DIC at a magnification of 400X.

Expression of dCK2 α -ts variants

Strains were grown in YPG for ~30 hr at the permissive temperature (29°C). An equal number ($\sim 10^7$) of cells of each strain were harvested and washed in 1X Phosphate Buffered Saline (PBS). Cells were solubilized with sample buffer [43] and boiled for 10 min. Cell extracts were normalized to total protein and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). One gel was stained with Coomassie, while the second was electrophoretically transferred to nitrocellulose. Western blot analysis was conducted using a polyclonal (rabbit) antibody raised against *Drosophila* CK2 holoenzyme [44] at a dilution of 1:1000 and secondary antibody (affinity purified goat-anti-rabbit IgG) coupled to alkaline phosphatase (Bio-Rad) at a dilution of 1:5000. Immunoblots were visualized using nitroblue tetrazolium (460 $\mu\text{g/ml}$) and 5-bromo-4-chloro-3-indolyl phosphate (16.5 $\mu\text{g/ml}$), and reactions were terminated by the addition of EDTA to a final concentration of 50 mM.

Yeast 2 Hybrid (Y2H) interactions

WT/ts alleles of dCK2 α were subcloned into the BamH1 and Xho1 sites of plasmid pGBT9, in which proteins are expressed as C-terminal fusions with the DNA-binding domain of yeast Gal4 [45]. dCK2 β/β' were expressed as C-terminal fusions with the activation domain of yeast Gal4, and were generated in plasmid pACT [46] as described previously [47,48]. Y2H assays were conducted in yeast strains PJ69-4a and PJ69-4 α [49] using a mating assay. Strain pJ69-4a was transformed with all pGBT9 constructs, whereas strain pJ69-4 α was transformed with the pACT constructs. Transformants were mated, selected on SM-Dex containing histidine, adenine, uracil and lysine. Expression of the reporter genes *HIS3*, *ADE2*, and *LacZ* was assayed on SM-Dex medium lacking histidine or adenine as previously described [48].

Molecular modeling of dCK2 α ^{ts} structures

Wild type and mutant proteins were modeled based on the known crystal coordinates of CK2. The Swiss PDB Viewer (www.expasy.org) and PyMOL (www.pymol.org) software packages were used to model the protein structure with the atomic coordinates of the human CK2 holoenzyme [50]. The corresponding residues of CK2 α were 'mutated' to identify vicinal residues affected by each mutation. The coordinates were obtained from the Protein Data Bank (ID code 1JWH).

Results

Selection of dCK2 α residues for site-specific mutagenesis

Four ts-alleles of *CKA2* have been described [21]. These are *cka2-7*, *cka2-8*, *cka2-11*, *cka2-12* and *cka2-13* (Fig. 1A). Based on the high conservation of CK2 α subunits, we identified residues that are conserved in dCK2 α , as candidates for mutagenesis. While four of these alleles

involved substitutions of two residues, one allele involved a single amino acid substitution. For example, *cka2-7* harbors the substitutions Ala¹⁹⁰Thr and Thr³³⁶Met, whereas *cka2-12* harbors the substitutions Ala¹⁹⁰Val and His²⁹⁴Tyr. Sequence alignments illustrated that Ala¹⁹⁰ is invariant in CK2 α subunits (Fig. 1B), whereas Thr³³⁶ or His²⁹⁴ are not conserved (not shown). For example, metazoan CK2 α subunits harbor a Pro in place of Thr³³⁶, which is located in vicinity of the C-terminus. In the crystal structure, Pro is not involved in structural stability of CK2 α or its ability to interact with CK2 β [38,50]. We therefore hypothesized that the Thr³³⁶Met substitution might not contribute to the ts-behavior. Similarly, His²⁹⁴ is unique to yeast CKA2p, and the corresponding position in other CK2 α subunits is either Arg (in CK2 α) or Asn (in CK2 α'). Since substitution of Ala¹⁹⁰ elicited ts behavior in two ts-alleles of CKA2, we targeted the corresponding residue, Ala¹⁷⁷ of dCK2 α Φ ty. 1B, and generated two variants, dCK2 α -A¹⁷⁷T and dCK2 α -A¹⁷⁷V.

The ts allele *cka2-8* (Fig. 1A) harbors two mutations Glu⁵¹Lys and Gly¹⁰²Asp (Fig. 1A). The former residue is not conserved in metazoan subunits, which harbor a Gln at this position. In contrast, Gly¹⁰² is invariant in metazoan CK2 α subunits (Fig. 1B). We therefore reasoned that Gly¹⁰² might (on its own) elicit ts behavior of *cka2-8*, and generated the variant dCK2 α -G⁸⁹D. The remaining two ts alleles are *cka2-11* and *cka2-13* (Fig. 1A). The former allele involves two substitutions, Asp²²⁵Asn and Glu²⁹⁹Lys, while the latter is characterized by a single substitution, Asp²²⁵Asn. Importantly, Asp²²⁵ is invariant in the Ser/Thr protein kinase family, and plays a role in the structural stability of the catalytic loop [51,52]. Since substitution of Asp²²⁵Asn in *cka2-13* was, by itself, sufficient to elicit ts behavior, we generated the variant dCK2 α -D²¹²N (Fig. 1A,B).

These variants were tested for their ability to complement the lethality of yeast lacking CK2 subunits (Fig. 1C), and employed the yeast strain RPG41-1a, which is deleted for the *CKA1* plus *CKA2* genes, and is rescued by a *URA3* marked plasmid carrying the wild type yeast *CKA2* gene. This strain was transformed with pESC-*LEU* plasmids that harbored the dCK2 α variants (see Table 1). Strains harboring both plasmids (*URA3-CKA2* plus *LEU2-dCK2 α*) were grown on SM-Gal media, to allow for expression of dCK2 α variants. The *URA3*-marked plasmid harboring *CKA2* was then evicted using 5FOA (see Materials and Methods). Plates were incubated at a sub-optimal temperature (25°C) to ensure the survival of the putative dCK2 α -ts strains. Under these conditions, survivors that had dropped the *URA3* marked plasmid were recovered from yeast strains YKP1-5 (see Fig. 1C, and data not shown). These rescued strains were named YKP6-10 (Fig. 1C and Table 1). As controls, we tested but found that strain RPG41-1a, by itself, or harboring the empty vector pESC-*LEU*, did not lead to any survivors following 5FOA treatment (data not shown), consistent with the essential role of CK2 for viability [9], and demonstrating that dCK2 α -constructs were necessary for rescue. The ability of wild-type dCK2 α to rescue yeast is consistent with previous studies [53], and suggests that the pESC-*LEU* vector is suitable for expression of dCK2 α , as also shown for *Xenopus* CK2 α [54]. Strains rescued by dCK2 α -variants exhibited a colony size comparable to that of a strain rescued by wild-type dCK2 α or yeast *CKA2* (data not shown), suggesting that these alleles are not functionally compromised at 25°C.

dCK2 α variants exhibit ts-behavior

We next tested strains rescued by dCK2 α variants for growth at the permissive temperature (PT, 29°C), and at higher non-permissive temperatures (NPT, 33, 35 and 37°C). As Fig. 2A shows, at 29°C yeast rescued by wild type dCK2 α exhibit growth comparable to the control strain RPG41-1a (expressing yeast *CKA2*). Furthermore, strains rescued by yeast *CKA2* or dCK2 α -WT displayed equivalent growth at 35 and 37°C; the slight growth deficit at 37°C was similar in both strains (Fig. 2A) and might reflect heat-shock effects of this higher temperature. These results suggest that dCK2 α -WT is not intrinsically temperature sensitive in yeast.

Similarly, strains YKP7-10 (rescued by dCK2 α variants) exhibited robust growth at 29°C (the PT), which appeared qualitatively similar to that of strains rescued by yeast *CKA2* or dCK2 α -WT (Fig. 2A). In contrast, strains YKP7, YKP8 and YKP9 displayed a complete growth-arrest at 33°C (not shown) or at 35°C (Fig. 2A), indicating that these variants were, indeed, temperature-sensitive. YKP10 (expressing dCK2 α -G⁸⁹D) exhibited significant, but incomplete, growth arrest at 37°C (Fig. 2A) indicating that it is the weakest ts-variant. A temperature higher than 37°C (such as 40°C) was not tested for dCK2 α -G⁸⁹D, because this temperature is, itself, non-permissive for growth of wild type yeast (data not shown), or those rescued by yeast *CKA2* or dCK2 α -WT (inset in Fig. 2C). Thus the rank order of temperature sensitivity is: yeast *CKA2* = dCK2 α -WT > dCK2 α -G⁸⁹D > dCK2 α -D²¹²N $\cong$ dCK2 α -A¹⁷⁷T > dCK2 α -A¹⁷⁷V (see inset in Fig. 2C). As controls, we tested strains *cka2-7*, *cka2-11* and *cka2-13*, and all exhibited ts-behavior and NPT's as previously described (Fig. 2A, and [21]). We also confirmed that expression of the dCK2 α variants was necessary for rescue, as no growth was detected in rich dextrose medium (YPD), which leads to repression of the *GAL1/10* promoters [55]. The weak growth of the dCK2 α -WT rescued strain in YPD (Fig. 1A) has been observed before [53], and might reflect low levels of a fully active protein.

We have previously found that expression of a catalytically null dCK2 α variant (Tik) in flies wild type for CK2 elicits dominant neural defects akin to loss of Notch signaling [28]. Because CK2 is a multimeric enzyme, we tested whether the dCK2 α -ts variants behaved in a dominant manner (in yeast), but found that in each case co-expression of wild type dCK2 α or yeast *CKA2* completely suppressed the temperature-sensitivity of the relevant strains (Fig. 2B), suggesting that these alleles are recessive. No such suppression was seen in strains YKP6-10 that were transformed with the empty expression vector, pESC-URA3 (data not shown).

It has been previously shown that depletion of CK2 results in 'pseudomycelial' cells [9]. Furthermore, ts-alleles of *CKA2* display cell-cycle arrest at the G1/S and G2/M transitions [21], whereas those of *CKA1* display enlarged cells due to defects in cell polarity [56], a phenotype also described for loss of CK2 in fission yeast [57]. We thus assessed the terminal phenotype of dCK2 α -ts rescued strains. Yeast with wild-type (endogenous) CK2 or those rescued by dCK2 α -WT display a normal cellular morphology at the PT (29°C) or at 37°C (Fig. 2C). Similar morphologies were observed for strains rescued by dCK2 α -ts variants at the PT of 29°C (data not shown). In contrast, when shifted to 37°C (the NPT), strains rescued by dCK2 α -A¹⁷⁷T and dCK2 α -G⁸⁹D displayed 'pseudomycelial' cells (Fig. 2C), a phenotype that recapitulates depletion of endogenous CK2 [9]. In contrast, strains rescued by dCK2 α -D²¹²N and dCK2 α -A¹⁷⁷V displayed enlarged cells, a phenotype akin to loss of *CKA1*. Thus, dCK2 α complements both, the cell cycle and cell polarity functions of *CKA2* and *CKA1*, respectively, and the terminal phenotype of strains rescued by dCK2 α -ts alleles mimic loss of endogenous (yeast) CK2 subunits.

Characterization of dCK2 α -ts variants

It has been previously shown that slow-growth upon lowered CK2 levels closely correlates with flocculation, a form of cell-cell aggregation [9,14]. We thus quantified these phenotypes in strains rescued by dCK2 α -ts variants to better assess their rescue efficiencies. At the PT (29°C), the doubling time of strain YKP6 (dCK2 α -WT) appear close to that of strain RPG41-1a (endogenous yeast CK2), and similar numbers were obtained for dCK2 α -A¹⁷⁷T, dCK2 α -D²¹²N and dCK2 α -G⁸⁹D (Fig. 3A). The reasons underlying the slightly higher extent of flocculation in the strains rescued by WT/ts-variants of dCK2 α is currently unknown, but it is possible that this reflects enzyme activity close to that which is rate limiting for cell proliferation. In contrast, strain YKP8 (rescued by dCK2 α -A¹⁷⁷V) exhibited a doubling time and degree of flocculation that were the highest (Fig. 3A, B). Given our observation that this variant does not elicit robust rescue at 29°C (the PT, see Fig. 2B), the possibility is high that

dCK2 α -A^{177V} is not fully active, and thus might be structurally compromised. It is important to note that a similar slow growth and flocculation defect has been described for the A^{190V} variant of yeast *CKA2* (*cka2-12*) even at 29°C [21].

The distinct NPT's of the *Ts* variants led us to assess whether this correlated to levels of expression/stability of the mutant proteins. We compared the steady-state expression levels of dCK2 in the *Ts* strains YKP7-10 to that of dCK2 α -WT (YKP6). The strains were grown in YPG to mid-log phase and cell extracts from equivalent number of cells were separated by SDS-PAGE and subjected to immunoblotting with anti dCK2 antibodies (Fig. 3C). This antiserum does not cross-react with the (yeast) *CKA2* subunit as evidenced by the absence of a band in extracts of RPG41-1a (Fig. 3C). When tested against rescued yeast, we found that levels of the dCK2 α -*ts* variants were comparable to that of dCK2 α -WT (Fig. 3C). Thus, the *ts*-behavior of dCK2 α variants and their rank order of sensitivity do not reflect attenuated expression levels or greater instability in yeast.

It has been previously shown that like the mammalian enzyme, CK2 purified from *Drosophila* embryos is an $\alpha_2\beta_2$ hetero-tetramer [58], and genetic analysis has shown that interaction of dCK2 α with dCK2 β is necessary for proper in vivo CK2 functions [59]. We therefore assessed for this interaction using the yeast interaction trap, as previously described by others and us [12,47,60,61]. Yeast expressing dCK2 α or dCK2 β , by themselves, did not elicit expression of the reporter genes *HIS3* or *ADE2* (as evidenced by their inability to grow in media lacking these amino acids), and the levels of *LacZ* induced under these conditions were indistinguishable from the 'baseline' (<10 Units, Fig. 3D). On the other hand, co-expression of dCK2 α plus dCK2 β elicited robust induction of all three reporter genes, indicative of a strong interaction, and similar results were obtained for the alternative subunit dCK2 β' (Fig. 3D, and data not shown). When tested with the dCK2 α -*ts* variants, we find that the D^{212N} and G^{89D} variants exhibited interaction (with dCK2 β or dCK2 β') close to that of the wild type protein, whereas dCK2 α -A^{177T} or dCK2 α -A^{177V} bound more strongly (see Discussion).

Discussion

The studies we report here provide direct evidence that residues altered in yeast CK2 *ts*-alleles elicit similar conditional destabilization of dCK2 α functions and thus constitute the first *ts*-variants of *Drosophila* CK2. Furthermore, these studies suggest that all of the yeast *CKA2*-*ts* alleles are, in fact, 'single hits', and further underscore the remarkable structural/functional conservation of these subunits. These dCK2 α variants efficiently rescue yeast lacking endogenous CK2 at the permissive temperature (Fig. 2), but this ability is neutralized at the higher non-permissive temperature. Quantitative analysis of growth rates, flocculation and expression levels (Fig. 3) suggest that these variants are not compromised for function at the lower temperature. Our observation that *ts*-variants recapitulate the well-described morphological defects that are associated with loss of yeast *CKA1p* or *CKA2p* (Fig. 2D) indicates that the dCK2 α -variants closely mimic the functions of endogenous (yeast) subunits. In addition, these variants display robust interaction with CK2 β (Fig. 3D) suggesting that holoenzyme formation is unlikely to be affected. While we have not directly assessed tetramer formation, we note that a robust two-hybrid interaction closely correlates to formation of the ($\alpha_2\beta_2$) holoenzyme in yeast and in vitro [12,53].

Based on these analyses, it appears that the D^{212N}, A^{177T} and G^{89D} variants of dCK2 α exhibit properties closest to the wild type protein, but are rendered non-functional at a higher temperature. This might not be the case for the dCK2 α -A^{177V} variant. Despite exhibiting the greatest temperature sensitivity, i.e., lowest NPT of any variant (32°C, see inset Fig. 2C), dCK2 α -A^{177V} does not appear to fully rescue yeast at lower temperatures (25 and 29°C), and exhibits an interaction with CK2 β that is stronger than that with the wild type protein. These

differences do not reflect effects of this substitution on stability/expression levels (Fig. 3C). In the case of human CK2 holoenzyme, Ala¹⁷⁹, which corresponds to Ala¹⁷⁷ (in dCK2 α) is not involved in the interaction of CK2 α with either the body or the tail of CK2 β [50], suggesting that substitution of Val at this site should have not influenced this interaction. It is reasonable to speculate that dCK2 α -A¹⁷⁷V is structurally compromised. Consistent with this possibility, it has been shown that the *cka2-12* allele, which harbors the A¹⁹⁰V substitution (see Fig. 1A), also exhibits the greatest temperature sensitivity [21]. Based on our studies, it thus appears that dCK2 α -D²¹²N, dCK2 α -A¹⁷⁷T and dCK2 α -G⁸⁹D exhibit properties of bona fide ts-alleles.

It has previously been shown that dCK2 α -WT purified from rescued yeast exhibits substrate specificity and kinetic constants that are comparable to protein purified from *Drosophila* embryos or from bacterial/insect cell expression systems [53,62-65]. However, a similar analysis on ts-variants has been difficult, since they lose activity upon partial purification from yeast (Bidwai, unpublished), perhaps, reflecting loss of chaperones such as HSP90 and/or CDC37, whose activities are required for maintaining CK2 (and other protein kinases) in a fully active state in vivo [66-70].

To better assess the impact of these substitutions on the ts-behavior, we used the atomic coordinates of human CK2 α [38,50], which is remarkably similar to dCK2 α [6,71]. In the crystal structure, human CK2 α consists of two domains, a β -strand rich N-terminal lobe and a C-terminal lobe rich in α -helices; the active site is located in the interfacial region (Fig. 4B). Importantly, the activation segment in human CK2 α is represented by sequences from Ile¹⁷⁴->Glu²⁰¹, (green box in schematic in Fig. 4A). Unlike cyclin-dependent kinases, such as CDK2, where the activation segment is conformationally stabilized upon binding of Cyclins, this segment is optimally positioned for catalytic activity of CK2 [50]. We find that Ala¹⁷⁷, which is located in the region between β -strands 8 and 9 (nomenclature from [50], Fig. 4A), itself, lies within the activation segment. The possibility is high that substitution of Ala¹⁷⁷ might impair the conformation of this critical region, and lead to a hypoactive kinase. Consistent with this prediction, replacement of Ala¹⁷⁷ with the bulkier Val residue generates a variant with the weakest complementation efficiency, whereas its replacement with Thr does not impair activity in a measurable manner (Fig. 2B). In contrast, neither Asp²¹² nor Gly⁸⁹ lie within the activation segment, and accordingly, variants of these sites exhibit normal rescue of yeast CK2 mutants, and mean generation times and interactions with CK2 β that closely mimic wild type dCK2 α (Fig. 3). Interestingly, the only null allele of dCK2 α identified to date (*Tik*) involves two substitutions (M¹⁶¹K and E¹⁶⁵D), which lie immediately outside the activation segment as well (Fig. 4A).

Molecular modeling confirms that despite their locations, Gly⁸⁹, Ala¹⁷⁷ and Asp²¹² lie in close proximity of the active site (Fig. 4B and data not shown). Given the evolutionary conservation of the catalytic core of Ser/Thr protein kinases [72,73], and that CK2 is closely related to the CDK's, we sought to determine whether the dCK2-ts alleles affect conserved residues. This is, indeed, the case, and we find that Ala¹⁷⁷ and Asp²¹² are invariant in *Drosophila*/human CDK2 as well as in the catalytic subunit of cAMP-dependent protein kinase (PKA, Fig. 4C). The behavior of Asp²¹² is of particular interest because this residue, which is located on α -helix F (nomenclature from [50], see Fig. 4A and B), is also conserved in human and *Drosophila* CDK2 and PKA, and this acidic residue is important for stability of the activation segment of PKA [51,74]. These findings suggest that substitution of Asp²¹² with Asn might engender collisions (of the amide) with vicinal residues. In the molecular model, amino acids within 6 angstroms of Asp²¹² are His¹⁴⁶, Arg¹⁵³ and Asp¹⁵⁴, and remarkably, all three residues are invariant in CDK2 and PKA. Thus, Asp²¹² represents a residue of CK2 α that is particularly sensitive for eliciting ts-behavior without perturbing its overall structure. Our studies suggest that the approach we describe should be of general applicability to other members of this family of enzymes.

The availability of these ts-alleles of dCK2 α will now permit genetic approaches in *Drosophila* by the replacement of the endogenous allele with these engineered variants. Given the applicability of *Drosophila* as a relevant model to study human diseases and developmental biology [35,75,76], such a resource will facilitate analysis of the roles of this important and conserved protein kinase. Finally, the conserved substitutions that elicit ts behavior should enable the targeting of similar residues in other protein kinases for which ts-alleles have not been forthcoming.

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Figure 1. Yeast *CKA2*-ts alleles

A) The substitutions in five ts-alleles of yeast *CKA2* are shown along with the corresponding residues in dCK2 α . Residues shown in bold are invariant in dCK2 α . B) Alignment of relevant segments of CK2 α subunits showing conservation of Gly⁸⁹, Ala¹⁷⁷, and Asp²¹². Sc-CKA1p has an insertion of 38 amino acids that is denoted as ‘^’. Abbreviations are: Hs, *Homo sapiens*; Xl, *Xenopus laevis*; Sc, *Saccharomyces cerevisiae*, and Dm, *Drosophila melanogaster*. C) Schematic of yeast screen.

Figure 2. dCK2 α variants exhibit ts-behavior

A) Yeast strains YKP6-10 rescued by the indicated dCK2 α variants were tested for growth on rich (YP) galactose media at the permissive temperature (29°C) and at higher non-permissive temperatures (35 and 37°C), or on rich (YP) dextrose media at 29°C. In each case, two independent clones were analyzed. In parallel, RPG41-1a (wild type *CKA2*) and three *cka2*-ts strains were also tested as controls. Note that the control strains YDH7 (*cka2-7*), YDH11 (*cka2-11*) and YDH13 (*cka2-13*) exhibit robust growth at 29°C, but are arrested at 35°C, a temperature higher than their minimum NPT. B) The indicated dCK2 α -variants were tested for ts behavior in the presence of wild type *CKA2* or wild type dCK2 α . C) Terminal morphologies of yeast cells expressing wild type *CKA2* or dCK2 α at 29 and 37°C. The phenotypes of strains rescued by dCK2 α -ts variants at the NPT (37°C); these cells exhibit morphologies akin to wild type cells at 29°C, but are not shown for simplicity.

Figure 3. Characteristics of dCK2α-ts variants

A and B) Mean generation time and percent flocculation were determined for the strains rescued by the indicated dCK2α variants, and for the control strain RPG41-1a (wild type *CKA2*). C) Immunoblot analysis to assess steady-state expression of dCK2α variants. Abbreviations are: RPG (strain RPG41-1a); WT, dCK2α-WT; A¹⁷⁷T, dCK2α-A¹⁷⁷T; A¹⁷⁷V, dCK2α-A¹⁷⁷V; D²¹²N, dCK2α-D²¹²N; and G⁸⁹D, dCK2α-G⁸⁹D. Note that this antibody does not cross-react with the product of the *CKA2* gene (RPG41-1a). D) Yeast two-hybrid interaction of dCK2α-ts variants with dCK2δ. Abbreviations are as in panel B. The reporter genes *HIS3* and *ADE2* were assayed by growth on minimal media and are depicted as none (—) or strong (+++). *LacZ* values are reported as Miller Units.

Figure 4. Modeling of substitutions in dCK2 α ts-variants

A) The locations of the substitutions eliciting ts-behavior are shown relative to the activation segment (AS, green box), and the relevant secondary structure motifs as described [50]. The M¹⁶¹ and E¹⁶⁵ substitutions are contained in the CK2 α null allele, *Tik*. B) Molecular model of CK2 α -D²¹²N. Note that the corresponding residue in human CK2 α is Asp²¹⁴ (right panel). C) Sequence alignments showing conservation of Ala¹⁷⁷ and Asp²¹² in human (Hs) and Drosophila (Dm) CK2 α , CDK2, and PKA. The numbers above the alignment refer to residues of dCK2 α , and green box depicts the 'Activation Segment'. D) Residues affected by the D²¹²N substitution in dCK2 α are in red, and the 'Motif Logo' shown above the alignment depicts conservation of these amino acids in CDK2 and PKA.

Table 1

S. cerevisiae plasmids and strains

Strain	Relevant chromosomal genotype	Plasmid genotype (URA3)	Plasmid genotype (LEU2)	Reference
RPG41-1a	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>CEN6/ARSH4 URA3 CKA2</i>	-----	[17]
YDH7	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	-----	<i>CEN6/ARSH4 LEU2 cka2-7</i>	[18]
YDH8	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	-----	<i>CEN6/ARSH4 LEU2 cka2-8</i>	[18]
YDH11	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	-----	<i>CEN6/ARSH4 LEU2 cka2-11</i>	[18]
YDH13	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	-----	<i>CEN6/ARSH4 LEU2 cka2-13</i>	[18]
YKP1	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>CEN6/ARSH4 URA3 CKA2</i>	<i>2μ LEU2 GALI^P-dCK2α-WT</i>	This study
YKP2	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>CEN6/ARSH4 URA3 CKA2</i>	<i>2μ LEU2 GALI^P-dCK2α-A¹⁷⁷T</i>	This study
YKP3	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>CEN6/ARSH4 URA3 CKA2</i>	<i>2μ LEU2 GALI^P-dCK2α-A¹⁷⁷V</i>	This study
YKP4	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>CEN6/ARSH4 URA3 CKA2</i>	<i>2μ LEU2 GALI^P-dCK2α-D²¹²N</i>	This study
YKP5	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>CEN6/ARSH4 URA3 CKA2</i>	<i>2μ LEU2 GALI^P-dCK2α-G⁸⁹D</i>	This study
YKP6	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	-----	<i>2μ LEU2 GALI^P-dCK2α-WT</i>	This study
YKP7	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	-----	<i>2μ LEU2 GALI^P-dCK2α-A¹⁷⁷T</i>	This study
YKP8	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	-----	<i>2μ LEU2 GALI^P-dCK2α-A¹⁷⁷V</i>	This study
YKP9	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	-----	<i>2μ LEU2 GALI^P-dCK2α-D²¹²N</i>	This study
YKP10	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	-----	<i>2μ LEU2 GALI^P-dCK2α-G⁸⁹D</i>	This study
YKP11	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>2μ URA3 GALI^P-dCK2α-WT</i>	<i>2μ LEU2 GALI^P-dCK2α-WT</i>	This study
YKP12	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>2μ URA3 GALI^P-dCK2α-WT</i>	<i>2μ LEU2 GALI^P-dCK2α-A¹⁷⁷T</i>	This study
YKP13	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>2μ URA3 GALI^P-dCK2α-WT</i>	<i>2μ LEU2 GALI^P-dCK2α-A¹⁷⁷V</i>	This study
YKP14	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>2μ URA3 GALI^P-dCK2α-WT</i>	<i>2μ LEU2 GALI^P-dCK2α-D²¹²N</i>	This study
YKP15	<i>MATa cka1-A1::HIS3 cka2-A1::TRP1</i>	<i>2μ URA3 GALI^P-dCK2α-WT</i>	<i>2μ LEU2 GALI^P-dCK2α-G⁸⁹D</i>	This study

All strains are derived from RPG41-1a, which carries the additional mutations *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ1 ura3-52*. The dCK2α-WT/mutant variants were expressed under control of the *GAL1* promoter (*GALI^P*) in the *2μ* expression vectors pESC-URA or pESC-LEU (Stratagene).