

Notes & Tips

## An activity-based two-hybrid system for the selective identification of substrates of protein kinases

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The yeast two-hybrid (Y2H)<sup>1</sup> system [1] has revolutionized the identification of protein–protein interactions and has had a tremendous impact on cell and developmental biology. In the Y2H system, two proteins are expressed as fusions with the DNA-binding domain (DB) of Gal4/LexA, the bait, and a transcription activation domain (AD), the prey. Interactions between the bait and the prey reconstitute a functional transcription factor that activates reporter genes that have been made responsive to binding by Gal4/LexA (reviewed in Refs. [2,3]). Since its initial development, the Y2H system has undergone many modifications, each of which has enabled unique applications. These adaptations include an array of DB and AD fusion vectors for expression of varying levels or types of fusion proteins, the use of multiple baits and reporters that enable simultaneous tests for specificity, the one- and three-hybrid systems to identify DNA-binding proteins and ternary interactions, and so forth. Although these adaptations have refined this approach, they do not enable discrimination of physiological targets of protein kinases that has generally required downstream biochemistry to confirm whether the interacting protein is also a target for phosphorylation. This last step is often rate limiting because it requires phosphorylation of recombinant proteins or peptides by purified kinase(s).

Our laboratory has employed the Y2H system extensively to identify physiological partners of CK2, a conserved Ser/Thr protein kinase from the fruit fly, *Drosophila* [4–7]. Such screens, using the catalytic ( $\alpha$ ) subunit of CK2, have identified an array of developmentally important

proteins. Analysis of the primary sequences of these proteins for the CK2 consensus site, (S/T)–(D/E/x)–x–(D/E), indicated that some of these were likely to be targets for phosphorylation, and this subsequently was confirmed via biochemistry. One of these studies involved the interactions of CK2 with three conserved basic helix–loop–helix (bHLH) repressors—M8, M7, and M5—derived from the *Enhancer of Split Complex (E(spl)C)* [7]. These proteins interacted strongly with CK2 $\alpha$  in the Y2H system and at the protein level, and they were phosphorylated at an invariant Ser residue. During attempts to map the phosphorylation site, we made the serendipitous finding that the CK2–M8 interaction was abolished upon phosphorylation. This result suggested that interaction and phosphorylation were coupled, thereby implicating the role of the active site of CK2 $\alpha$ . Although these studies suggested that the CK2–M8 interaction might reflect a prototypical enzyme–substrate interaction, it was not applicable to other proteins identified in our screens such as the regulatory subunit of CK2, CK2 $\beta$ , and the ribosomal protein, rpL22, whose interaction and phosphorylation domains were separable [8,9]. Therefore, we hypothesized the presence of two classes of CK2 targets: those where interaction is independent of the active site (class 1, CK2 $\beta$  and rpL22) and those where the active site was required (class 2, M8/7/5). In addition, we reasoned that catalytically inactive CK2 $\alpha$  might distinguish class 2 proteins from class 1 proteins. We found that this is indeed the case, and we tested three novel proteins using this simplified discriminatory assay.

To test this hypothesis, we employed a variant of CK2 that substitutes Lys66 with Met (referred to as CK2 $\alpha$ -K<sup>66</sup>M). This substitution eliminates phosphotransferase functions and thus catalytic activity [10]. Both wild-type CK2 $\alpha$  and CK2 $\alpha$ -K<sup>66</sup>M were expressed as AD fusions. The DB fusions employed in our initial test included the regulatory CK2 $\beta$  subunit as a control and the bHLH repressors

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<sup>1</sup> Abbreviations used: Y2H, yeast two-hybrid; DB, DNA-binding domain; AD, activation domain (AD); bHLH, basic helix–loop–helix; E(spl)C, enhancer of split complex.

M8, M7, and M5 encoded by the E(spl)C. In addition, we used two variants of M8: a nonphosphorylatable variant (M8S<sup>159</sup>A) and one that mimics the constitutively phosphorylated protein (M8S<sup>159</sup>D).

We found that both isoforms of CK2 $\alpha$ , the active and the inactive variant, interact robustly with CK2 $\beta$ , a class 1 protein (Fig. 1). The independence of the CK2 $\alpha$ – $\beta$  interaction with respect to the functional state of the active site is consistent with the three-dimensional structure of the CK2 ( $\alpha\beta$ ) holoenzyme in which the  $\alpha$ – $\beta$  contacts are distal to the active site [11]. In contrast, significant differences are observed when class 2 proteins are analyzed with the two forms of CK2 $\alpha$ . As we reported previously, wild-type CK2 $\alpha$  interacts robustly with E(spl)M8/7/5 (Fig. 1). Surprisingly, however, CK2 $\alpha$ -K<sup>66</sup>M displayed a significantly attenuated interaction with these bHLH proteins, suggesting that a functional active site is vital for these interactions. In each case, the levels of *LacZ* for the CK2 $\alpha$ -K<sup>66</sup>M interactions decreased between 5- and 10-fold as compared with wild-type CK2 $\alpha$ . The attenuated interactions of CK2 $\alpha$ -K<sup>66</sup>M are not due to attenuated expression or instability (in yeast) given that the interactions of CK2 $\alpha$  or CK2 $\alpha$ -K<sup>66</sup>M with CK2 $\beta$  are virtually identical (Fig. 1). Thus, the highly attenuated interactions of CK2 $\alpha$ -K<sup>66</sup>M with M8/7/5 are likely to reflect the inability of this inactive protein to recognize these targets. Based on our observation that phosphorylation disrupts the CK2–M8 interaction, we also tested for interactions with CK2 $\alpha$ -K<sup>66</sup>M with M8S<sup>159</sup>A/D. We found that the attenuated interactions of M8S<sup>159</sup>A with CK2 $\alpha$  is further decreased when tested with CK2 $\alpha$ -K<sup>66</sup>M. In contrast, M8S<sup>159</sup>D, a variant that mimics the constitutively phosphorylated state in vivo [12], does not interact differentially with either CK2 $\alpha$  isoform.

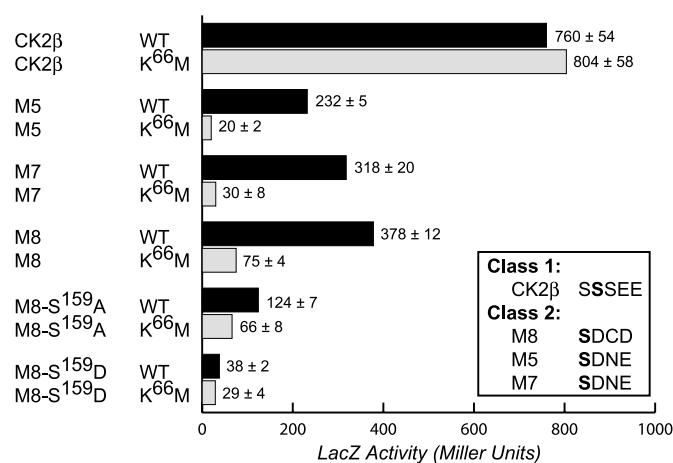


Fig. 1. Interaction of CK2 $\alpha$  and CK2 $\alpha$ -K<sup>66</sup>M with CK2 $\beta$  and E(spl)bHLH proteins. Yeast EGY048 was transformed with plasmids expressing LexA–CK2 $\beta$ /bHLH fusions in combination with AD–CK2 $\alpha$  (WT, solid bars) or AD–CK2 $\alpha$ -K<sup>66</sup>M (gray bars). Transformants were grown in galactose medium, and the levels of *LacZ* were determined in triplicates. *LacZ* activity is expressed in Miller units. The inset shows the known CK2 phosphorylation site(s) in CK2 $\beta$  and E(spl)M5/7/8.

We further tested the possibility that CK2 $\alpha$ -K<sup>66</sup>M can selectively identify class 2 proteins. For this purpose, we employed two proteins (Hairy and Daughterless) that are predicted to be CK2 substrates, one protein (Deadpan) that we recently reported to be a CK2 target [13], and one protein (Groucho) that is not predicted to be a target. Consistent with our studies that CK2 phosphorylates Deadpan, we found that the interaction of this protein with CK2 $\alpha$ -K<sup>66</sup>M is attenuated approximately threefold when compared with wild-type CK2 $\alpha$ . Interactions with CK2 $\alpha$ , albeit weak ones, were also observed for Hairy and Daughterless (Fig. 2). The lower levels of *LacZ* induced in these binary combinations directly reflect the strong positive role of CK2 $\beta$  that is, however, dispensable for interactions of CK2 $\alpha$  with M5/7/8 [6]. For example, we reported that purified monomeric CK2 $\alpha$  interacts weakly with and phosphorylates Deadpan, whereas the holoenzyme is enhanced approximately 10-fold [13]. A similar situation also occurs for the CK2–Hairy interaction (our unpublished data). In each case where CK2 $\alpha$ -K<sup>66</sup>M was tested, its interactions were attenuated approximately two- to threefold as compared with the wild type. On the other hand, a similar analysis with Groucho elicited only baseline induction of *LacZ* ( $\leq 5$  Miller units in this system) with either wild-type or catalytically dead CK2, raising the possibility that this protein is not targeted by CK2. The observed interactions (Hairy, Daughterless, and Deadpan) and noninteraction (Groucho) with CK2 $\alpha$  are of particular interest because only the former three proteins contain a consensus site(s) for CK2 phosphorylation (Fig. 2, inset).

In summary, we have shown that the functionality of the active site of CK2 predictably influences its ability to interact with proteins whose phosphorylation is a prerequisite for complex formation. Given the common evolutionary

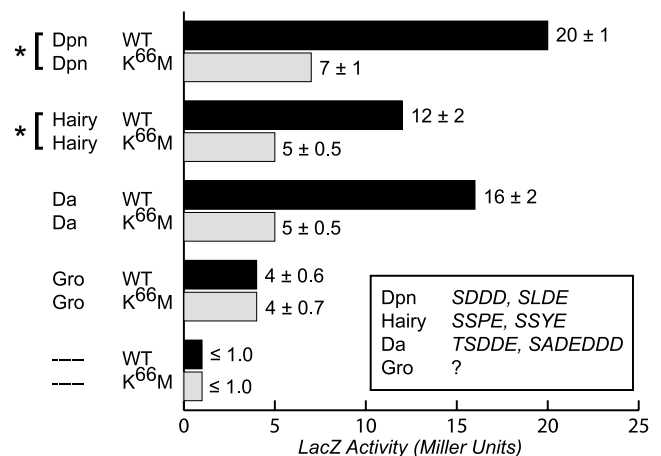


Fig. 2. Interaction of CK2 $\alpha$  and CK2 $\alpha$ -K<sup>66</sup>M with known/putative CK2 substrates. Yeast EGY048 was transformed with plasmids expressing LexA fusions in combination with AD–CK2 $\alpha$  (WT, solid bars) or AD–CK2 $\alpha$ -K<sup>66</sup>M (gray bars). Dpn, Deadpan; Da, Daughterless; Gro, Groucho. Transformants were grown in galactose medium, and the levels of *LacZ* were determined in triplicates. *LacZ* activity is expressed in Miller units. The inset shows the CK2 consensus site(s) in Dpn, Da, and Hairy. Asterisks denote proteins confirmed to be phosphorylated by CK2.

relationships of Ser/Thr protein kinases and that this group of enzymes are structurally similar [11], our studies suggest that the strategy described here should be generally applicable. This approach can be readily adapted to existing versions of the Y2H system such as the dual-bait system [14], where interactions between a protein or a random cDNA library and two variants of CK2 (or other protein kinases) can be assayed simultaneously. Given the amenability of this approach to high-throughput analysis, this strategy might be generally applicable to other regulatory enzymes, such as kinases and phosphatases, and may accelerate the elucidation of signal transduction pathways and the identification of the site(s) of phosphorylation.

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