Use of the two-hybrid system to identify protein–protein interaction temperature-sensitive mutants: application to the CDK2/p21Cip1 interaction

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ABSTRACT

We describe the application of the two-hybrid system to the identification of protein–protein interaction temperature-sensitive mutants. We applied this strategy to the interaction between the human CDK2 cell cycle regulator and the p21Cip1 regulatory subunit. A library of randomly generated CDK2 mutant proteins was screened for interaction with p21Cip1 at different temperatures. This approach resulted in the isolation of single point mutations in CDK2 causing temperature-sensitive interaction with p21Cip1. Our results demonstrate that the two-temperature two-hybrid screen is an efficient approach for the rational design and screening of protein–protein interaction conditional mutations.

Since the discovery of yeast cell division cycle (cdc) mutants in the early 70s, the field of cell cycle research has largely benefited from the use of temperature-sensitive (ts) mutations (1). In yeasts, various mutants defective in the progression of the cell cycle at the restrictive temperature have been isolated and have proven to be powerful tools for the identification of the genes required for cell cycle control (2,3). Temperature-sensitive mutants have also been identified in cultured mammalian cells and turned out to be very useful for the study of the role of oncogenes and tumour suppressors in growth control (4,5). The absence of an efficient method to generate and characterise ts mutations in vertebrate proteins has greatly limited the use of such mutants in mammals, although as demonstrated with SV40 large T or p53, conditional mutations are very useful for the study of the role of oncogenes and tumour suppressors in growth control (4,5). The absence of an efficient method to generate and characterise ts mutations in vertebrate proteins has greatly limited the use of such mutants in mammals, although as demonstrated with SV40 large T or p53, conditional mutations are very useful for the study of the role of oncogenes and tumour suppressors in growth control (4,5).

The yeast-based two-hybrid system is becoming a widely used investigation method for protein–protein interaction studies (6,7). It has been successfully used to identify proteins that bind to a protein of interest, to assay interaction between mutant proteins (including thermosensitive forms), and to search for peptides that bind to a protein of interest (8–11). Here, we describe a new application of the two-hybrid system, for the identification of protein–protein interaction ts mutants. We applied this strategy to the interaction between CDK2, one of the key enzymes that regulate the eukaryotic cell cycle (12), and p21Cip1, a potent CDK inhibitory protein induced by p53 in response to DNA damage (13).

To identify CDK2 mutants temperature-sensitive for the interaction with p21Cip1 we first constructed a randomly generated cDNA library of CDK2 mutants. Low fidelity polymerase chain amplification was performed on the human CDK2 wild-type cDNA template using an unbiased dNTP ratio and a Taq polymerase lacking proof reading activity (14). PCR reactions were performed with pBS-CDK2 plasmid, using oligonucleotide primers 5′-CATGGAGAATTTCAAAAAG-3′ (containing an internal EcoRI restriction site as shown underlined) and 5′-GGAGACAAAAAGCTGGACC-3′. PCR was carried out in a volume of 100 µl which included 120 ng of plasmid DNA, 60 pmol each primer, 5 mM MgCl2, 5 U Taq polymerase (Promega), 10 µl of 10× PCR buffer (500 mM KCl, 100 mM Tris–HCl pH 8.3, 0.1% gelatine, 1 mM DTT) and variable concentrations of dNTP (Pharmacia). Two PCR reactions were performed using either dGTP or dTTP at a concentration of 20 µM, while the other dNTPs were used at a normal concentration (200 µM). A total of 40 cycles was employed with 30 s denaturation at 95°C, 30 s annealing at 58°C, 1 min polymerisation at 72°C. The amplification products (mutated CDK2 cDNAs) were then digested with EcoRI and BamHI and directionally cloned into the same sites of the pGAD two-hybrid vector (Clontech) in frame with sequence encoding Gal4 transcriptional activation domain. The resulting cDNA library (~2 × 10⁸ independent clones before amplification with <5% empty vectors) was amplified in Escherichia coli and used to transform yeast strain YGH-1 (kindly provided by G. Hannon, Cold Spring Harbor) carrying plasmid pGBT9-p21 encoding human p21Cip1 in frame with Gal4 DNA binding domain. Double transformants were selected on SD synthetic medium plates lacking Leu, Trp and His, and containing 5 mM 3-amino-triazol. The leucine and tryptophan prototrophies were provided by the pGAD and the pGBT9 vectors, respectively. The activation of the His3 reporter-gene relied on the reconstitution of the Gal4 transcription factor through the interaction between CDK2 and p21. Only 25% of the transformants carrying both plasmids were able to grow on the selective media lacking histidine (His* colonies), indicating that the remaining 75% transformants harboured mutations that fully abolished the interaction between CDK2 and p21Cip1. These were possibly frameshift, premature

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termination or mutations severely impairing the overall structure of CDK2. To select for ts mutations, His+ colonies that grew normally at the permissive temperature of 25°C were replica-plated and tested for their ability to form colonies at the restrictive temperature of 37°C. About 5% of the His+ colonies displayed a growth defect at 37°C indicative of a p21Cip1–CDK2 heat-sensitive interaction. In contrast, the positive control strain pGAD–CDK2/p21Cip1, which carries wild type CDK2, grew at all temperatures. As shown in Figure 1A, the CDK2 mutants isolated, are specifically impaired for the binding to p21Cip1 or are more widely affected.

Figure 1. Identification of single point mutations in CDK2 causing temperature-sensitive interaction with p21Cip1. (A) Temperature sensitivity of the CDK2 mutant/p21Cip1 interaction. CDK2 wild-type or mutants, in frame with the Gal4 activation domain, were co-expressed with p21Cip1 fused to the Gal4 DNA binding domain, in yeast strain YGH1 (MATα, ura3-52, his3-200, ade2-101, lys2-801, trpl-901, leu2-3, can1, gal4-542, gal80-538, ura3::GAL1-LacZ, lys2::GAL1-UAS-GAL1-Tata-HIS3). Cells (2 × 10^6) were spotted onto four selective medium plates lacking Trp, Leu, His, and supplemented with 5 mM 3-AT (3-amino-1,2,4-triazole, Sigma). Plates were then incubated at 25, 30, 33 or 37°C for 5 days, respectively. (B) CDK2 mutations resulting in temperature-sensitive CDK2/p21Cip1 interaction. Codon changes and subsequent residue substitution are indicated.

REFERENCES


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