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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 loss of function is easily achieved upon shift-up of the cells grown at 32.5–39°C.

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 unbalanced dNTP ratio and a *Taq* polymerase lacking proof reading activity (14). PCR reactions were performed with pBS-CDK2 plasmid, using oligonucleotide primers 5′-CATGGAGAATTCCAAAAG-3′ (containing an internal *Eco*RI restriction site as shown underlined) and 5′-GGAACAAAAGCTGGAGC-3′. PCR was carried out in a volume of 100 µl which included 120 ng of plasmid DNA, 60 pmol each primer, 5 mM $MgCl_2$, 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 *Eco*RI and *Bam*HI 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 (~2×10^4 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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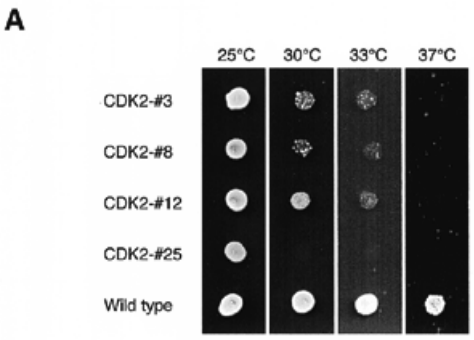

B

Mutant	CDK2#8	CDK2#3	CDK2#12	CDK2#25
Base	428	512	568	821
Wild-type seq.	CTA Leu143	CCT Pro171	CTG Leu189	CCG Arg274
Mutant seq.	CAA Gln143	CAT His171	CCG Pro189	CAG Gln274

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 YGH-1 (*MATα, ura3-52, his3-200, ade2-101, Lys2-801, Trp1-901, leu2-3, can^r, gal4-542, gal80-538,* URA3::GAL1-LacZ, LYS2::GAL1UAS-GAL1Tata-HIS3). Cells (2×10^4) 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 3, 3, 4 and 5 days, respectively. (**B**) CDK2 mutations resulting in temperature-sensitive CDK2/p21Cip1 interaction. Codon changes and subsequent residue substitution are indicated.

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/pGBT9-p21 YGH1 which carries wild type CDK2 grew similarly at both permissive and restrictive temperatures, while the negative control strains lacking pGAD–CDK2 and/or pGBT9-p21 did not grow at any temperature. The temperature sensitivity of the interaction between p21Cip1 and four of the CDK2 mutants identified in this screen, was then assayed at various temperatures. As shown in Figure 1A, the CDK2 mutants analysed displayed differential temperature sensitivity for the interaction with p21Cip1. Although all the mutants interacted at 25°C and failed to interact at 37°C, clear differences were observed at the intermediate temperatures (see for example mutant CDK2-#12). The assay is therefore sufficiently sensitive to discriminate between CDK2 mutants exhibiting different temperature sensitivity.

Sequencing of the full length CDK2 mutants cDNAs revealed that >50% contain isolated point mutations. The four mutants shown in Figure 1 contain a single point mutation resulting in only one amino acid exchange. All the substitutions are non-conservative with, for instance, polar residues replacing hydrophobic (L143Q) or basic (R274Q) residues. Mapping of the mutations on the CDK2 sequence indicates that all substitutions target residues that are very well conserved among the members of the CDK family, from yeast to man (data not shown). Interestingly, the mutation R274Q that alters CDK2 binding to p21Cip1 (mutant CDK2#25) is equivalent to the mutation R283Q, previously identified in the cdc18-13 allele of the CDC28/CDC2 budding yeast CDK and shown to cause ts cell cycle progression (15). This mutation in CDK2 (R274Q) may therefore alter not only p21Cip1 binding but also CDK2 catalytic activity. Future studies will be required to determine which, among the different CDK2 ts mutants isolated, are specifically impaired for the binding to p21Cip1 or are more widely affected.

The two-temperature two-hybrid screen, that we have developed, is a very efficient method to screen a large number of randomly generated mutant proteins for ts mutations. In our study, screening of ~3000 colonies resulted in the isolation of 35 ts mutants. The isolated ts mutations may either affect residues that directly participate in the interaction or disrupt local structural elements that help support the actual contact domains. The selection of mutants that retain the capacity to interact at the permissive temperature eliminates relatively uninteresting mutations such as truncations, frameshifts or mutations severely impairing the three dimensional structure of the protein. The method described in this article could be applied to any biological question that requires the rational generation and use of protein–protein interaction conditional mutants. The two-temperature two-hybrid screen will likely prove to be the method of choice for the identification and characterization of these conditional mutations in vertebrate proteins.

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REFERENCES

1 Reed,S., Hutchinson,C.J. and MacNeill, S. (1995) In Hutchinson,C. and Glover,D.M. (eds) *A Brief History of the Cell Cycle. Cell Cycle Control*. IRL Press at Oxford University Press. Oxford.
2 Hartwell,L.H., Culotti,J., Pringle,J.R. and Reid,B.J. (1974) *Science* **183**, 46–51.
3 Nurse,P., Thuriaux,P. and Nasmyth,K. (1976) *Mol. Gen. Genet.* **146**, 167–178.
4 Jat,P.S. and Sharp,P.A. (1989) *Mol. Cell. Biol.* **9**, 1672–1681.
5 Pietenpol,J.A., Lengauer,C., Jordan,J., Kinzler,K.W. and Vogelstein,B. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 8391–8394.
6 Chien,C., Bartel,P.L., Sternglanz,R. and Fields,S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9578–9582.
7 Fields,S. and Song,O. (1989) *Nature* **340**, 245–246.
8 Fields,S. and Sternglanz,R. (1994) *Trends Genet.* **10**, 286–292.
9 Yang,M., Wu,Z. and Fields,F. (1995) *Nucleic Acids Res.* **23**, 1152–1156.
10 Warbrick,E. (1997) *Structure* **5**, 13–17.
11 Gekakis,N., Saez,L., Delahaye-Brown, A.-M., Myers,M.P., Sehgal,A., Young,M.W. and Weitz,C.J. (1995) *Science* **270**, 811–815.
12 Morgan,D.O. (1995) *Nature* **374**, 131–134.
13 Elledge,S.J., Winston,J. and Harper,W. (1996) *Trends Cell Biol.* **6**, 388–392.
14 Leung,D.W., Chen,E. and Goeddel,D.V. (1989) *Technique* **1**, 11–15.
15 Lorincz,A.T. and Reed,S.I. (1986) *Mol. Cell. Biol.* **6**, 4099–4103.