Application of the Green Fluorescent Protein as a Reporter for Ace1-Based, Two-Hybrid Studies

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ABSTRACT

The two-hybrid system in Saccharomyces cerevisiae is a genetic approach for the detection of protein-protein interactions in vivo. This technology relies on the activity of separated DNA-binding and transactivation domains of specific transcription factors to reconstitute an active transcription factor complex if interacting proteins are fused to these domains. Interactions are consequently detected through the activity of reporter genes. The two-hybrid technology has been successfully applied for the determination of interactions between numerous proteins of several organisms. Conventional reporter systems, such as the β-galactosidase from Escherichia coli, suffer from a variety of drawbacks, including the requirement for external substrates. In this report, we describe an alternative version of the two-hybrid system using the combined advantages of the copper-inducible transcription factor Ace1 together with the yeast metallothionein gene CUP1 and the green fluorescence protein from aquatic invertebrates as reporters. This technique allows the copper-dependent monitoring of protein-protein interactions in living yeast cells.

INTRODUCTION

Since its introduction in 1989, the two-hybrid system in Saccharomyces cerevisiae has become a widely distributed technology for the detection of protein-protein interactions in vivo (7). Recently, we developed a two-hybrid approach that is based on the activity of the yeast transcription factor Ace1 (14). Ace1 stimulates transcription of the CUP1 gene through binding to regulatory sequences within the CUP1 promoter (8,16). CUP1 encodes the yeast metallothionein, which tightly chelates intracellular copper and other metal ions, protecting the cell against copper toxicity (9). The binding of Ace1 to DNA is dependent on the presence of copper ions, because copper can induce a conformational change in the amino terminus of Ace1, allowing the protein to interact with DNA (8).

To monitor protein-protein interactions with the Ace1-based, two-hybrid system, the coding sequences of the respective proteins were fused to those of the Ace1 DNA-binding domain and the transactivation domain. These hybrid proteins were expressed in yeast reporter strains. Positive interactions were detected by the survival of cells on copper-containing media due to the expression of genomic CUP1 or through a copper-dependent expression of the β-galactosidase (β-gal) linked to the CUP1 promoter (14).

For the elimination of false positives that occur frequently in the two-hybrid system, several reporter genes under control of different promoters have been developed (1). Recently, the green fluorescent protein (GFP) from Aequorea victoria has been described as a novel reporter for two-hybrid studies (4,13). The GFP makes yeast cells strongly fluorescent without causing toxicity (15). Light-stimulated GFP fluorescence does not require any cofactors, external substrates or additional gene products. Detection of GFP can be performed in living cells instead of fixed samples and without cell lysis.

In this report, we describe an improved Ace1-based, two-hybrid system, which is more efficient than the existing one (14). In contrast to the conventional two-hybrid systems, the reporter gene activity is inducible by copper ions, which is of value for certain applications, e.g., drug screening. Moreover, we used the advantages of the GFP as a readout for monitoring protein-protein interaction. This combination allows a copper-dependent detection of interacting proteins in living yeast cells.

MATERIALS AND METHODS

Yeast Strains, Transformation and Gene Replacement of ACE1

Transformation of yeast cells followed the protocol of Klebe and coworkers (11). Yeast transformants were selected and cultivated onto SD synthetic medium (2% glucose and 0.67% yeast nitrogen base without amino acids) supplemented with essential amino acids and nucleotides. The copper-resistant yeast strain DBY747 (MATα ura3-52 his3-171 trp1-289 leu2-3,-112 CUP1) was used as a host for the two-hybrid analyses. A gene-replacement cassette was constructed, which contained the ACE1 coding sequence substituted by the TRP1 selection marker. The genomic ACE1 allele was disrupted in strain DBY747 by homologous recombination at the NcoI site within the ACE1 promoter and the Nhel site within the ACE1 terminator regions present on the disruption cassette. Colonies were selected on SD medium lacking tryptophan. This gave rise to strain TFY118-1 (MATα ura3-52 his3-171 trp1-289 ace1::TRP1 leu2-3,-112 CUP1). Correct gene-replacement events were verified by loss of copper resistance and by polymerase chain reaction (PCR) with primers flanking the insertion sites. Amplified sequences were analyzed by agarose gel electrophoresis. The plasmid pTY99, carrying a CUP1 promoter-GFP reporter (cycle 3 mutant gene) (5) construct, was linearized with BsrEI and integrated into the leu2 locus of TFY118-1. The transformants were selected on medium that lacked leucine. This strain was named ITH5 (MATα ura3-52 his3-171 trp1-289 ace1::TRP1 leu2-3,-112 LEU2::pTY99 CUP1).

GFP Assay

The derivatives of the GFP reporter strain ITH5 carrying the two-hybrid
plasmids were grown overnight at 30°C in SD medium. Cells were diluted with SD medium to a cell density of OD_{600nm} = 0.5, and 100 µL of the suspension were transferred to opaque 96-well plates together with various CuSO_4 concentrations. The microplates were covered by sticking strips and a lid and incubated for several hours on a shaking platform at 30°C. The on-line registration of GFP activity was monitored upon excitation at 405 nm, and the whole cell fluorescence was measured at 510 nm using the Victor 1420 Multilabel Counter Luminescence Spectrophotometer (EG&G Wallac, Turku, Finland). The copper-induced GFP fluorescence was expressed as arbitrary units regarding the cellular background fluorescence, which was detected before the addition of copper.

RESULTS AND DISCUSSION
Development of a Novel Ace1-Based, Two-Hybrid System and Verification by Metallothionein Expression

As an alternative to conventional two-hybrid systems, we have developed a two-hybrid approach based on the activity of the yeast transcriptional activator Ace1 (14). This system suffers from inconveniences due to the necessity of a genomic integration of the Ace1 DNA-binding domain (Ace1BD) plasmid. We now show that this can be avoided using the Ace1-based, two-hybrid vectors pTY137 (carrying Ace1BD) and pTM114 [carrying the Ace1 activation domain (Ace1AD)], which contain their own origins of replication (see Figure 1). Both plasmids can be transformed simultaneously into yeast cells. To enhance the probability of nuclear import of the prey hybrid protein, the Ace1AD was fused to the nuclear localization sequence of the simian virus 40 (SV40) large T antigen (10).

The system was tested with the homodimerization of the yeast regulatory subunit of the cyclic AMP-dependent protein kinase encoded by the BCY1 gene (3,17). The coding sequences for the amino terminal localized dimerization part of Bcy1 (17) were fused to the sequences of Ace1BD and Ace1AD using plasmids pTY137 and pTM114 (Figure 1). Plasmids were co-transformed in the yeast strain ITH5, which carries several tandemly amplified copies of the CUP1 gene. The strain ITH12, expressing both Ace1BD-Bcy1 and Ace1AD-Bcy1 fusion proteins, was resistant to 200 µM copper, whereas the strains ITH11 (expressing Ace1BD and ACE1p, promoter of the ACE1 gene. Detailed description of the expression plasmid constructions are available from the corresponding author.

Figure 1. Key vectors for the two-hybrid system carrying the coding sequences of the (a) Ace1BD and (b) Ace1AD. GAPDHp, promoter of the glyceraldehyde-3-phosphate dehydrogenase gene; NLS, nuclear localization sequence of the SV40 large T antigen; CYC1t, terminator sequences of the cytochrome c1 oxidase gene; and ACE1p, promoter of the ACE1 gene. Detailed description of the expression plasmid constructions are available from the corresponding author.
Ace1AD alone), ITH16 (containing Ace1BD-Bcy1 and Ace1AD) and ITH17 (containing Ace1BD and Ace1AD-Bcy1) did not grow at concentrations of more than 10 µM copper (Figure 2). Thus, the dimerization of Bcy1 reconstituted a functional Ace1 protein, which then induced the transcription of CUP1, detected by an enhanced copper resistance of yeast cells. This was in accordance with our previously published data (14). The Ace1 two-hybrid system described in this report did not require a genomic integration of the bait vector and therefore enabled a co-transformation of both plasmids.

We propose that our system will also be suitable for a screening with activation domain tagged libraries to identify proteins interacting with a given bait. The transforms carrying Ace1BD-bait and Ace1AD-prey plasmids, or optionally, the diploid cells obtained by the mating-selection method of a two-hybrid screening (2), can be spread directly onto agar plates containing CuSO$_4$. Only those cells expressing interacting proteins can endure the toxicity of copper ions, detected by the growth monitoring on copper-containing plates. To test this, we transformed ITH5 with the Ace1-Bcy1 containing plasmids and spread the cells onto plates supplemented with 30–100 µM copper. Only those cells expressing both fusion proteins grew on copper medium (not shown). Because interacting clones are identified by a growth selection, this system is highly sensitive as stated by Le Douarin et al. (12). Modulating the stringency of the CUP1 selection by different concentrations of copper might allow the discrimination between strong or weak interactions in a screening procedure at an early step.

The GFP Is a Reporter for Ace1-Based, Two-Hybrid Analyses

The detection of the enzymatic activity of classical reporters for quantitative two-hybrid studies, such as the $\beta$-gal from *Escherichia coli*, usually requires cell lysis and the addition of substrates. Furthermore, reporter activities can only be monitored at a given endpoint. In this report, we describe the application of a copper-inducible GFP expression as an alternative reporter system allowing the measurement of protein-protein interaction directly in living yeast cells.

For our studies, we used the strain ITH5 containing a stable genomic integrated copy of a CUP1 promoter-GFP reporter fusion. The copper-regulated functional GFP expression was validated by overexpression of the entire ACE1 coding sequence, as present on a 2-µm-derived vector (not shown).

The application of the copper-dependent GFP expression in two-hybrid analyses, using the homodimerization of Bcy1 as an example, was tested with the plasmids described above. Plasmids

![Figure 2. Induction of metallothionein expression through homodimerization of Bcy1 in the Ace1-based, two-hybrid system.](image2)

Equal numbers of cells from yeast strains ITH11 (expressing Ace1BD and Ace1AD), ITH16 (expressing Ace1BD-Bcy1 and Ace1AD), ITH17 (expressing Ace1BD and Ace1AD-Bcy1) and ITH12 (expressing Ace1BD-Bcy1 and Ace1AD-Bcy1) were spotted onto SD plates containing the indicated CuSO$_4$ concentrations and incubated for 3 days at 30°C.

![Figure 3. (Lower panel) GFP activity in the yeast strains ITH17 (expressing Ace1BD and Ace1AD-Bcy1, dark grey bars), ITH16 (expressing Ace1BD-Bcy1 and Ace1AD, bright gray bars) and ITH12 (expressing Ace1BD-Bcy1 and Ace1AD-Bcy1, black bars). Cells were incubated with the indicated CuSO$_4$ concentrations in microplates. The GFP activity was determined as the difference of yeast background fluorescence and the fluorescence obtained after a 4-h incubation with copper. The means and standard deviations (error bars) from at least 6 experiments are shown. (Upper panel) Growth increase of yeast strains ITH17, ITH16 and ITH12 during a 4-h incubation in microplates. Following the fluorescence detection, cells of each strain were collected from the wells and transferred into micro-square cuvettes. The cell density was measured photometrically at 600 nm. Concentration at t = 0 was OD$_{600nm}$ = 0.5.](image3)
containing the Ace1BD-Bcy1 and Ace1AD-Bcy1 hybrids were co-transformed together or with plasmids carrying the Ace1BD and the Ace1AD alone into ITH5. Figure 3 shows that copper induced an enhanced GFP signal up to fourfold in strain ITH12 expressing both Ace1-Bcy1 proteins compared to the noninduced culture. In contrast, the strains ITH16 and ITH17 expressing either the Ace1BD-Bcy1 hybrid or the Ace1AD-Bcy1 hybrid alone showed a reduced fluorescence after addition of copper as compared to the untreated cells. These results reflect the relationship between the GFP expression and the survival of yeast cells under copper stress. Due to the lack of CUP1 expression, the cells containing noninteracting Ace1 fusion proteins were sensitive to copper treatment, implying that these cells entered a cell cycle arrest, which also contributed to a reduced GFP signal. Cells expressing interacting Ace1 fusion proteins produced metallothionein and are therefore growth-resistant to copper. Figure 3 shows that the cell growth was more reduced in copper-treated cells of strain ITH12 compared to untreated cells. Therefore, the strongly enhanced GFP signal in these cells was not dependent on a growth increase but on the copper-dependent stimulation of GFP expression.

The combination of CUP1 and GFP expression should also allow for distinguishing strong from weak interactors. As reported earlier, it is inappropriate to use the quantitative readout of only one reporter to determine the strength of interaction (6). In our system, the growth of yeast strains expressing low-affinity interacting proteins on copper will be much more depressed than that of cells containing strongly interacting pairs. Furthermore, the GFP signal in these cells is greatly reduced compared to strains expressing proteins with a high-binding affinity.

Our data demonstrate that the copper-dependent GFP reporter expression allowed the measurement of Bcy1 dimerization using the Ace1-based, two-hybrid system. The detection of GFP fluorescence does not require lysis of yeast cells. This allows the monitoring of reporter gene activity as a consequence of time. To prove this, ITH12 cells were treated with 100 µM CuSO₄, and fluorescence was measured after different time points. The GFP signal in these cells increased with the incubation time, whereas the untreated cells produced only a slightly enhanced GFP fluorescence (not shown).

Taken together, our data have shown that the GFP and the endogenous CUP1 reporter in combination with the copper-inducible Ace1 transcription factor, allowed an efficient detection of protein-protein interaction in living yeast cells. Our system is a simple and fast approach avoiding cell lysis and the addition of substrates for detection of enzymatic activities. The commercially available displayGREEN™ Two-Hybrid System (Display Systems Biotechnology, Vista, CA, USA) uses the GFP reporter in combination with lexA and the acidic activator peptide B42. In this system, the bait, prey and reporter genes are located on three different 2-µm-derived plasmids. To avoid recombination events, our system uses vectors from different origins of replication (see Figure 1).

Finally, we would like to stress that two-hybrid systems can be setup to screen for compounds interfering in the interaction between therapeutic relevant proteins, which has been demonstrated recently (18). We propose that the described copper-inducible, Ace1-based, two-hybrid system is a very useful approach for the development of high-throughput screening assays. The expression of the reporter gene in our system is solely initiated by the addition of copper. Therefore, the incubation of cells with an inhibitory drug followed by the addition of copper should result in a clearly reduced GFP expression. Conversely, the reporter gene in non-inducible systems is already expressed during the whole fermentation process of yeast cells before the addition of inhibiting compounds. Due to the already existing level of the reporter proteins, the inhibitory effects of such compounds could hardly be detected. Work is in progress to examine the qualification of the Ace1 system for drug screening purposes.

REFERENCES

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Generation of Vector-Insensitive Dig-Labeled Probes from Large Cosmid Library Inserts Using PCR

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ABSTRACT

We have devised a general method for producing vector-insensitive probes from clones in which insert DNA (ca. 40 kb) could not be amplified in one piece nor be excised from the vector sequence. The method involves PCR and vector-specific primers in combination with restriction digestion and ligation. It yields specific PCR