Reconstitution of human RNA interference in budding yeast

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ABSTRACT

Although RNA-mediated interference (RNAi) is a widely conserved process among eukaryotes, including many fungi, it is absent from the budding yeast Saccharomyces cerevisiae. Three human proteins, Ago2, Dicer and TRBP, are sufficient for reconstituting the RISC complex in vitro. To examine whether the introduction of human RNAi genes can reconstitute RNAi in S. cerevisiae, genes encoding these three human proteins were introduced into S. cerevisiae. We observed both siRNA and siRNA- and RISC-dependent silencing of the target gene GFP. Thus, human Ago2, Dicer and TRBP can functionally reconstitute human RNAi in S. cerevisiae, in vivo, enabling the study and use of the human RNAi pathway in a facile genetic model organism.

INTRODUCTION

The RNA interference (RNAi) pathway is an evolutionarily conserved mechanism of gene regulation (1). Discovered as a biological response to double-stranded RNA (dsRNA) in the nematode Caenorhabditis elegans (2), the RNAi pathway has been shown to be present in many eukaryotes, ranging from fission yeast to human.

The yeast Saccharomyces cerevisiae is a ‘model’ model organism with an impressive collection of resources for expression and mutational studies, and dynamic molecular and cell-biological phenotyping. Although analogous functions have been uncovered in closely related species (3), the budding yeast S. cerevisiae completely lacks any analog to the RNAi pathway (4), suggesting that it may provide an ideal test-bed for the molecular genetic characterization of the exogenous human RNAi pathway.

Gene silencing via RNAi requires the RNA-induced silencing complex (RISC), a large ribonucleoprotein complex composed of three proteins, Argonaute-2 (Ago2), Dicer and HIV-1 transactivating response (TAR) RNA-binding protein (TRBP) (5–6). The RISC complex cleaves pre-miRNA and selectively loads a guide miRNA onto Ago2. The three purified proteins Ago2, Dicer and TRBP were previously shown to successfully reconstitute RISC in vitro without any cofactors or chaperones (7–10). Together, these results raise the possibility that the RNAi pathway could be established in S. cerevisiae to enable detailed molecular genetic studies.

MATERIALS AND METHODS

Plasmid construction

(i) Human Ago2, Dicer and TRBP expression plasmids. Human Ago2, Dicer and TRBP cDNAs (11) individually cloned into Advanced Gateway Destination Vectors (12) by Gateway recombination cloning (Invitrogen) to produce the galactose-inducible expression plasmids pAG413Gal-Ago2, pAG416Gal-Dicer and pAG415Gal-
TRBP, respectively. All plasmids are CEN-based and under the control of GAL1 promoter with different auxotrophic markers. All constructs were sequence-verified. (ii) Silencing antisense constructs. GFP (S65T) and Ade2 of antisense orientation were cloned into pAG424Gal to produce silencing constructs pAG424Gal-AS-GFP or pAG424Gal-AS-Ade2. These are 2-micron plasmids under GAL1 promoter. The Ade2 antisense construct was used as a negative control.

Yeast strains and methods

LPY3498 (MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 ESA1) (13) was used to create a GFP-expressing RNAi reporter strain. The GFP(S65T)-KanMX6 module from pFA6a was integrated at the endogenous TDH3 locus in LPY3498 to create a wild-type strain (WT) used in this study [MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 ESA1 TDH3::GFP(S65T)-KanMX6]. The WT strain was sequentially transformed with three plasmids (described above), each bearing one of the human RISC-encoding genes, and selected using auxotrophic markers to generate the "ADT" strain. All yeast strains were grown at 30°C according to the standard protocol. Transformation was carried out as previously described (14).

Reverse transcription–PCR

Total RNA was isolated using the hot phenol method. Reverse transcription (RT) reactions were performed with total RNA and oligo(dT) primer using Superscript III according to the manufacturer's instructions (Invitrogen). PCR reactions were assembled in 50 µl with 2 μl RT reaction and the following gene-specific primers: Ago2 forward, 5'-AGCGCCAGTGACGGAAAGCT; Ago2 reverse, 5'-AGGTGCGGAAACGTGCTCC; Dicer forward, 5'-CCAGCTGTGGGGAGAGGGCCT; Dicer reverse, 5'-TCAGGGACTAGGCACAGG; GC; TRBP forward, 5'-CGCATCTCGTCCAGGGGC; TRBP reverse, 5'-GCCGGCTGGGTGGACAGT; TC; ACT1 forward, 5'-CGTTCCAATTTACGCTGG; TT; ACT1 reverse, 5'-AGTTTGATCAATACCCGGCAG. After 30 cycles, an aliquot was removed and analyzed by a 1.5% agarose gel electrophoresis. DNA was visualized by EtBr staining.

Northern blot analysis

Small RNA blots were performed using 10–15 µg total RNA per lane and carbodiimide-mediated cross-linking to the membrane (15), with the following DNA probes: 5’-TATGCAGGGGAACTGTGAT radio-labeled at their 5’ termini for S. cerevisiae U6 small nuclear RNA (SNR6; GenBank Accession No. X12565); a full-length GFP cDNA probe radiolabeled by random priming for GFP siRNA.

Flow cytometry

Each strain was inoculated in prototrophic selection media with 2% raffinose and grown overnight. Fresh cultures were then seeded from the overnight cultures and cells were grown to log-phase with either 2% glucose (non-inducing) or 2% galactose (inducing). Cells were analyzed using FACSCalibur (BD Biosciences) and data were processed with CellQuest Pro (BD Biosciences).

RESULTS AND DISCUSSION

In an attempt to reconstitute the human RNAi system in budding yeast, three human RNAi genes were introduced into S. cerevisiae, together with the appropriate silencing and reporter constructs. Human Ago2, Dicer and TRBP cDNAs were individually cloned and placed under the control of the inducible GAL1 promoter using the Gateway recombination cloning system. For use in silencing, antisense GFP was cloned under the GAL1 promoter; antisense Ade2 was used as a negative control (Figure 1A). A GFP-expressing reporter strain was created by integrating the GFP(S65T)-KanMX6 module into the endogenous TDH3 locus (Figure 1B). The GFP-expressing reporter strain was sequentially transformed with plasmids bearing each of the three human RNAi gene constructs, and the expression of each gene under galactose-induction conditions was confirmed by RT-PCR (Figure 2A). Expression of RISC genes was not observed when the yeast strains were not induced in galactose. This Ago2/Dicer/TRBP (ADT) strain was transformed with plasmids bearing the antisense GFP silencing constructs, followed by northern blot detection of GFP siRNA (Figure 2B). Introduction of human Ago2, Dicer and TRBP was sufficient to generate GFP siRNA in S. cerevisiae. Strains expressing all possible pairwise combinations of two RISC components (Ago2/TRBP, Ago2/Dicer and Dicer/TRBP) were also constructed. The GFP siRNA biogenesis was observed in the strain lacking Ago2 and the strain lacking TRBP, but not in the strain lacking Dicer, indicating that Dicer is necessary for siRNA biosynthesis.

Figure 1. A schematic diagram showing the silencing antisense constructs and the GFP reporter strain. (A) GFP or Ade2 were each separately cloned under control of GAL1 promoter in the antisense orientation to generate silencing antisense constructs. (B) GFP reporter strain was created by homologous recombination of GFP(S65T)-KanMX6 module into TDH3 locus.
biogenesis and that Ago2 and TRBP are not (Figure 2B).

Flow cytometric analysis was next performed to assess whether the introduced human RNAi genes are capable of silencing GFP gene expression. When the ADT strain expressing antisense-GFP silencing construct was induced with galactose, a significant decrease in the GFP fluorescence intensity was observed, indicating that human RNAi system was successfully reconstituted in *S. cerevisiae* (Figure 3).

Silencing effects were not observed either under the uninduced condition or when an antisense-ADE2 construct was used in place of the antisense-GFP construct. Strains expressing all possible pairwise combinations of two RISC components (Ago2/Dicer and Dicer/TRBP) did not exhibit silencing effects (Table 1). Taken together, our results show that the three human genes are necessary and sufficient for reconstitution of the human RNAi system in *S. cerevisiae*.

In this study, we used antisense GFP as a silencing construct. Although hairpin sequences can also be used for this purpose, the GFP hairpin RNA transcribed under the *GAL1* promoter may lack a structure that is required for Dicer-mediated processing (16–18). Although RNA polymerase III promoters are often used for the transcription of shRNAs in mammalian cells (19–21), the yeast U6 RNA promoter is ill-defined (22). Antisense GFP RNA should hybridize with the endogenous GFP transcript, and thus generate substrate double-stranded RNA for Dicer-mediated cleavage. Indeed, GFP siRNA was detected by northern blot (Figure 2) as was target gene silencing (Figure 3). The silencing effect of antisense GFP alone (without the RNAi genes) was negligible (Figure 3, bottom).

Recently, Drinnenberg et al. (3) reported the reconstitution of RNAi in *S. cerevisiae* by introducing Dicer and
Table 1. Comparison of silencing effects of different combination of RISC components

| RISC components | Glucose condition | Galactose condition |
|-----------------|-------------------|--------------------|
| Ago2/Dicer/TRBP  | 455.2 ± 3.5       | 91.8 ± 0.8         |
| Ago2/TRBP       | 430.1 ± 3.9       | 422.7 ± 4.7        |
| Ago2/Dicer      | 389.6 ± 2.6       | 392.0 ± 3.6        |
| Dicer/TRBP      | 456.5 ± 4.6       | 446.3 ± 4.1        |

The GFP-expressing *S. cerevisiae* strain was transformed with the indicated combination of RISC components and the GFP-silencing constructs. GFP gene silencing was determined by flow cytometric analysis under either non-inducing (glucose) or inducing (galactose) condition. Values indicate the mean fluorescence intensity ±SEM.

Argonaute of *Saccharomyces castellii*. They identified a novel class of Dicer protein present in *S. castellii* which—unlike other known Dicer genes in *Schizosaccharomyces pombe*, plants and animals—has a novel class of Dicer protein present in budding yeast. The reconstituted human RNAi system could facilitate functional dissection of human RNAi proteins and domains such as the helicase domain of Dicer and Argonaute of *S. cerevisiae*.

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