

Construction of a Novel Set of Control Preys for the Yeast Split-Ubiquitin System

by

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Abstract

In order to investigate the physical interaction between two membrane proteins, we usually use the yeast split-ubiquitin system (YSUS). In the YSUS, two control prey constructs, pDL2-Alg5 and pAI-Alg5, which were developed based on the yeast *ALG5* gene, have been prepared as negative and positive controls for detection of protein interaction, respectively. These control prey constructs are available only when yeast Alg5p protein does not physically interact with any other protein. However, our previous study demonstrated that human orthologous protein, hDPGS, interacted with itself. This fact suggests the possibility that yeast Alg5p would also interact with itself. If so, both control preys might not be suitable for analysis of Alg5p. Therefore, we tried to develop a novel negative control prey that does not interact with Alg5p at all. For this purpose, we focused on our recent observation that hDPGS exhibited no interaction with truncated hDPGS, which contains only a region from the N-terminus to the second hydrophobic region (HR2). In order to replace a portion of the full-length Alg5p with that of truncated Alg5p (Alg5HR2p), which corresponds to truncated hDPGS, a coding region of Alg5p from the N-terminus to HR2 was amplified by PCR, and ligated with pPR3-C vector to transform *Escherichia coli* for molecular cloning. This manipulation yielded the negative control prey construct (pDL2-AG5HR2), which would express Alg5HR2p-NubG fusion protein in yeast cells. In addition, to create the positive control prey construct, combinatorial PCR with primers for introduction of point mutation was carried out using pDL2-AG5HR2 as a template. After PCR, the resultant fragment was ligated with pPR3-C vector to transform *E. coli*, yielding the positive control prey construct (pAI-AG5HR2), which would express Alg5HR2p-NubI fusion protein in yeast cells. Using these constructs as controls, physical interaction of Alg5p was analyzed using the YSUS. As a result, it was revealed that Alg5p did not interact with Alg5HR2p-NubG, but did interact with Alg5HR2p-NubI. This observation indicates that the novel prey constructs work well as negative and positive controls.

Keywords: Split-ubiquitin system, Control prey, Physical interaction

1. Introduction

The study of protein-to-protein interaction has become important more and more, because elucidation of the relationship among various proteins is one of key points for understanding a number of biological phenomena. As a quite useful tool for this purpose, the yeast two-hybrid system was devised, which can detect physical interaction between two proteins *in vivo*. However, it has been demonstrated not to be suitable for analysis of membrane proteins, as it forces to artificially localize two proteins of interest into the nucleus of the yeast cell.

To overcome this disadvantage, the yeast split-ubiquitin system (YSUS) was later developed for analysis of physical interaction between two membrane proteins¹⁻⁴⁾. This system can detect protein-to-protein interaction naturally occurring

on the rough endoplasmic reticulum (rER) membrane. Using the YSUS, various physical interactions among membrane proteins, including enzymes and subunits of an enzymatic complex residing on the rER membrane, have been revealed⁵⁻⁸⁾.

We also have used the YSUS, and investigated physical interactions among human glycosyltransferases and related proteins that play a role in biosynthesis of dolichol-linked oligosaccharide (DLO) on the rER membrane⁹⁻¹²⁾. Recently, we analyzed physical interactions of human dolichol-P-glucose synthase (hDPGS)¹³⁾. From a result of the growth examination using co-transformants with hDPGS bait and hDPGS1 prey, we noticed that hDPGS physically interacted with itself to form homo-dimer¹³⁾. As yeast Alg5p is orthologous to hDPGS, they share similar primary structure and conformation with each other¹⁴⁾. Hence, it is possible that Alg5p might interact with Alg5p-NubG, which is produced by the negative control prey pDL2-Alg5 prepared in the YSUS. As the negative and positive control preys in the YSUS are presupposed to exhibit no physical interaction of Alg5p with

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Table I The PCR primers used in this study. Additional sequences containing a *Sfi* I- cleavage site are shown in lowercase letters.

Primer name	Nucleotide sequence	Purpose
AG5 fw	5'-tgtaatggccattacggccATGAGAGCGTTGAGATTCCTGATTG-3'	Cloning of <i>ALG5HR2</i>
AG5HR2 rv	5'-gcctttggccgagcgccgaTGTGTCTTTGATAGACCTGATGCCG-3'	Cloning of <i>ALG5HR2</i>
NubI fw	5'-CTTTGACCGGTAAAACCATAACATTGGAAGTTGAATC-3'	Mutagenesis from <i>NubG</i> to <i>NubI</i>
NubI rv	5'-GATTCAACTTCCAATGTTATGGTTTTACCGGTCAAAG-3'	Mutagenesis from <i>NubG</i> to <i>NubI</i>
NUB rv	5'-TTACATGACTCGAGGTCGACATCC-3'	Cloning of <i>ALG5HR2-NubI</i>
dl2xn seqfw	5'-GTCGAAAATCAAGACAAGG-3'	Cloning of <i>ALG5HR2-NubI</i>

any membrane protein, these observations suggest that the conventional set of these control preys could not be suitable for analysis of DPGS derived from any organism.

Therefore we attempted to construct the novel set of control preys in order to entirely exclude the possibility that yeast Alg5p interacts with any protein, including itself. For this purpose, we focused on the observation that hDPGS did not interact with truncated hDPGS which has a region from the *N*-terminus to the second hydrophobic region (HR2)¹³. Hence, our strategy for creating the novel preys was to exchange full-length *ALG5* portion (1002 bp) in pDL2-Alg5 and pAI-Alg5 for truncated AG5HR2 portion (741 bp) that encodes a region from the *N*-terminus to HR2 of Alg5p.

2. Experimental Methods

2.1 Construction of the negative control prey (pDL2-AG5HR2)

In order to produce the DNA fragment which encodes a region from the *N*-terminus to HR2 of yeast Alg5p, PCR with AG5_fw and AG5HR2_rv primers (Table 1) was performed using yeast genomic DNA as template by KOD ver.2 DNA polymerase (TOYOBO) [condition: 94 °C 2 min, (98 °C 10 sec, 60 °C 30 sec, 68 °C 1 min) by 36 times, 68 °C 2 min]. Amplified 741bp fragment and prey vector pPR3-C, which contains NubG, were digested with *Sfi* I restriction enzyme (TOYOBO) at 50 °C overnight, purified and ligated with each other by DNA ligase (TOYOBO) at 16 °C for 1 hour. Transformation of *Escherichia coli* JM109 with ligation mix by a method developed by Inoue *et al.*¹⁵ yielded several colonies on ampicillin-containing LB plates. Plasmids were prepared from cultures inoculated from colonies, digested with *Bam* HI and *Eco* RI restriction enzymes (TOYOBO) at 37 °C for 2 hours, and subjected to analysis by agarose gel electrophoresis¹⁶. The plasmids which yielded a 741bp fragment were selected as candidates of pDL2-AG5HR2 and subjected to DNA sequencing analysis.

2.2 Construction of the positive control prey (pAI-AG5HR2)

Using pDL2-AG5HR2 as template, combinatorial PCR¹⁷ was conducted in order to convert *ALG5HR2*-NubG portion to *ALG5HR2*-NubI portion as follows. PCR with dl2xn_seqfw and NubI_rv primers was carried out under the following condition: 94 °C 2 min, (98 °C 10 sec, 60 °C 30 sec, 68 °C 1 min) by 36 times, 68 °C 2 min. This yielded a head part of *ALG5HR2*-NubI portion (841 bp). On the other hand, PCR with NubI_fw and NUB_rv primers was carried out under the following condition: 94 °C 2 min, (98 °C 10 sec, 60 °C 30 sec, 68 °C 30 sec) by 36 times, 68 °C 1 min, to produce a tail part of *ALG5HR2*-NubI portion (101 bp). Both parts were mixed,

heated at 100 °C for 5 min and cooled down to room temperature so that the two fragments hybridized with each other. Using the mixture as template, the second round PCR was performed with dl2xn_seqfw and NUB_rv primers under the same condition as amplification of the head part, to produce the full-length fragment of *ALG5HR2*-NubI portion (942 bp). Amplified 942 bp fragment and pPR3-C were digested with *Bam* HI and *Sal* I restriction enzymes (TOYOBO) at 37 °C for 3 hours, purified and ligated with each other by DNA ligase (TOYOBO) at 16 °C for 1 hour. Transformation and selection of desired recombinant plasmid pAI-AG5HR2 were performed by a standard cloning method¹⁶ described above.

2.3 Validation of pDL2-AG5HR2 and pAI-AG5HR2

The bait construct, pBT-C-Alg5 was used for co-transformation of the *Saccharomyces cerevisiae* NMY51 strain, together with a conventional set of control preys (pDL2-Alg5 and pAI-Alg5) or the novel set of control preys (pDL2-AG5HR2 and pAI-AG5HR2). The transformation of the yeast NMY51 cells was carried out by the standard method¹⁸. The co-transformants obtained on the synthetic dextrose (SD) medium lacking leucine and tryptophan (SD-LW) were then subjected to the growth examination on the SD medium lacking leucine, tryptophan and histidine (SD-LWH) and SD medium lacking leucine, tryptophan, histidine and adenine (SD-LWHA), according to the manual supplied by Dualsystems Biotech (www.dualsystems.com).

3. Results and Discussion

3.1 Molecular cloning of pDL2-AG5HR2 and pAI-AG5HR2

The YSUS takes advantage of the property of the yeast ubiquitin protein, in which *C*-terminal half portion (Cub) spontaneously associates with wild type *N*-terminal half portion (NubI) to form split-ubiquitin (Fig. 1A). To detect the physical interaction between two membrane proteins, NubG which is no longer able to spontaneously associates with Cub was devised by point mutagenesis of NubI (Fig. 1B). Despite abolishment of spontaneous association with Cub, NubG still can form split-ubiquitin only when it locates near Cub (Fig. 1C). This proximity between NubG and Cub occurs when the Nub-fused membrane protein physically interacts with the Cub-fused membrane protein (Fig. 1C). Therefore, the “negative” control prey protein fused with NubG must not interact with any bait protein fused with Cub (Fig. 1B). In contrast, the “positive” control prey protein fused with NubI should always form split-ubiquitin together with any bait protein fused with Cub (Fig. 1A). As mentioned above, we

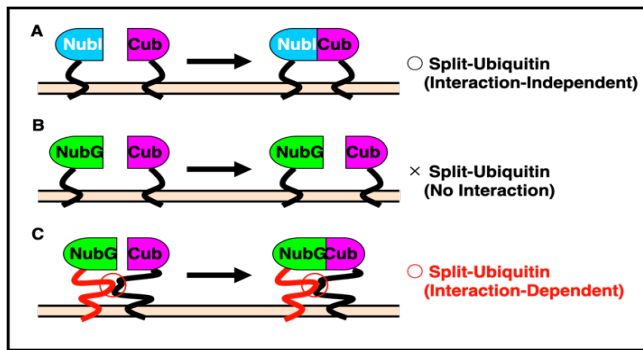


Fig. 1 The principal of the yeast split-ubiquitin system (YSUS). A. bait protein fused with Cub vs. Alg5p-NubI expressed by pAI-Alg5. B. bait protein fused with Cub vs. Alg5p-NubG expressed by pDL2-Alg5. C. bait protein fused with Cub vs. prey protein fused with NubG.

suspected that Alg5p fused with NubG expressed from pDL2-Alg5 construct, might interact with Alg5p fused with Cub. In order to exclude this possibility, we had an idea that a region from the *N*-terminus to HR2 of Alg5p (Alg5HR2p) should be used rather than full-length Alg5p as the negative control prey. This idea was based on our observations that full-length hDPGS interacted with itself, and that a region from the *N*-terminus to HR2 of hDPGS (hDPGSHR2) no longer interacted with full-length hDPGS¹³.

At the same time, full-length Alg5p portion expressed from positive control prey pAI-Alg5 also should be replaced with the same Alg5HR2p for comparison. Hence, we constructed both pDL2-AG5HR2 and pAI-AG5HR2 as the

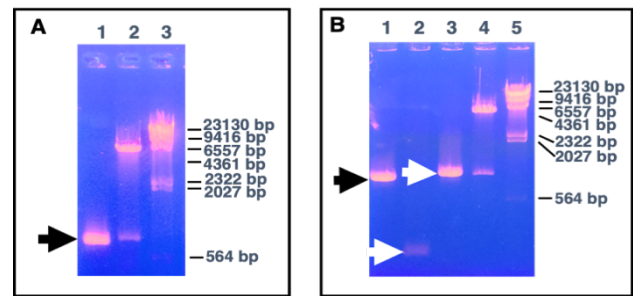


Fig. 2 A. Electrophoresis of PCR product described in section 2.1 (lane 1), pDL2-AG5HR2 after digestion with *Bam* HI and *Eco* RI (lane 2) and λ/*Hind* III size marker (lane 3). B. Electrophoresis of combinatorial PCR products described in section 2.2 (head part; lane 1, tail part; lane 2, entire; lane 3), pAL-AG5HR2 after digestion with *Bam* HI and *Sal* I (lane 4) and λ/*Hind* III size marker (lane 5).

novel set of control preys.

Firstly, pDL-AGHR2 was produced by PCR cloning. The 741 bp fragment containing *AG5HR2*-coding region was successfully amplified by PCR (a black arrow of lane 1 in Fig. 2A) and cloned into pPR3-C vector via *Sfi* I sites (lane 2 in Fig. 2A).

Subsequently, pAI-AG5HR2 was constructed by introducing G13I substitutional mutation into *Nub* gene to convert *NubG* to *NubI*. For this purpose, combinatorial PCR using pDL2-AG5HR2 containing *AG5HR2-NubG*-coding region as template was carried out. PCR with dl2xn_seqfw and NubI_rv primers generated 810 bp fragment containing head part of *AG5HR2-NubI* (a black arrow of lane 1 in Fig. 2B),

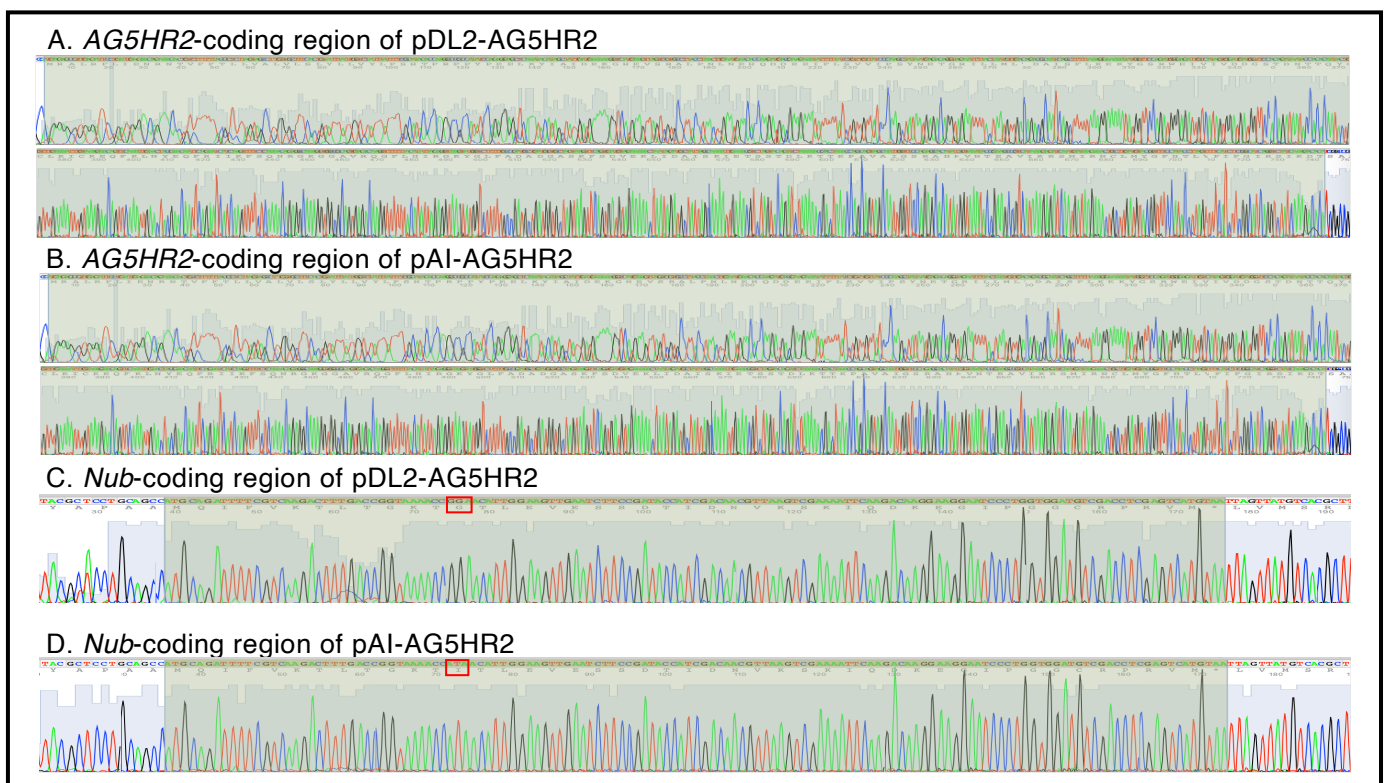


Fig. 3 Sequencing analysis. Nucleotide sequences of pDL2-AG5HR2 and pAI-AG5HR2. Sequencing reaction for analysis of *AG5HR2* or *Nub* portion was primed with dl2xn_seqfw (A and B) or Nub_fw primer (C and D), respectively. The dl2xn_seqfw or Nub_fw primer anneals against sequences upstream from *AG5HR2*- or *Nub*- initiation codon, respectively. Each coding region is highlighted in yellow color.

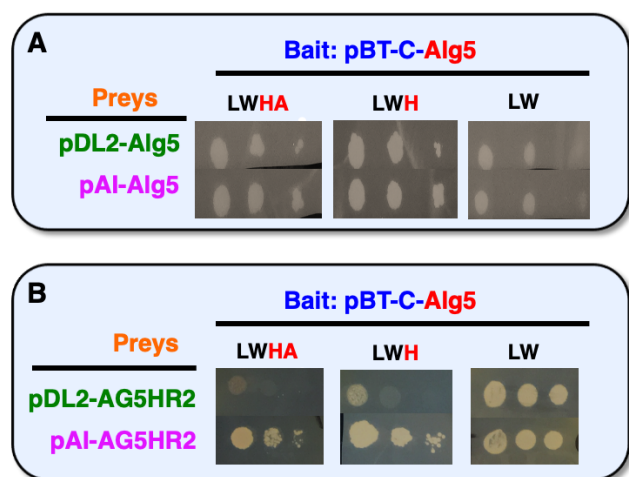


Fig. 4 Growth examination of co-transformant with pBT3-C-Alg5 (A and B). In A, the conventional control preys (pDL2-Alg5 and pAI-Alg5) were used, while the novel control preys (pDL2-AG5HR2 and pAI-AG5HR2) were used in B.

while that with NubI_fw and NUB_rv primers generated 110 bp fragment containing tail part of *AG5HR2-NubI* (a white arrow of lane 2 in Fig. 2B). Hybridization of both fragments and subsequent PCR with dl2xn_seqfw and NUB_rv primers produced 910 bp fragment including entire *AG5HR2-NubI*-coding region (a white arrow of lane 3 in Fig. 2B). The resulting fragment was cloned into pPR3-C vector via *Bam* HI and *Sal* I sites. (lane 4 in Fig. 2B). Both prey constructs obtained were then subjected to nucleotide sequencing and respectively confirmed as proper recombinant clones (Fig. 3A). Hence, nucleotide sequences of *AG5HR2*-coding portion derived from pDL2-AG5HR2 and pAI-AG5HR2 perfectly accorded (Fig. 3A and Fig. 3B), and those of *Nub*-coding portion matched with each other except only the thirteenth codon (indicated by red boxes in Fig. 3C and Fig. 3D).

3.2 The novel control prey set properly works on the analysis of yeast *Alg5p*

In order to test the nature of the set of prey constructs newly prepared above, they were individually co-introduced into yeast NMY51 cells together with bait construct pBT3-C-Alg5 and co-transformants were subjected to growth examination on SD-LWH and SD-LWHA media.

When the conventional set of prey constructs were used in such co-transformation and growth examination, co-transformants with pDL2-Alg5 grew on both selective media, as well as those with pAI-Alg5 (Fig. A). This result obviously indicated that Alg5p-NubG from pDL2-Alg5 physically interacts with Alg5p-Cub from pBT3-C-Alg5, via a portion of Alg5p. The possibility that Alg5p might form a homodimer has been already reported by Drake *et al.*¹⁹⁾ and therefore our result strongly supports this possibility.

In contrast, co-transformants with pDL2-AG5HR2 barely grew on both selective media, although those with pAI-AG5HR2 normally grew on both media (Fig. 4B). These observations indicated that C-terminus of Alg5HR2p must be indeed oriented towards cytoplasmic side, and that Alg5HR2p

itself should no longer physically interact with full-length Alg5p.

Taken together, in case of analyses of the physical interaction of yeast Alg5p, pDL2-AG5HR2 and pAI-AG5HR2 constructs successfully functioned as negative and positive controls, respectively.

4. Conclusion

Since we started using the Y2H system twenty years ago, our question as to whether the Alg5p portion expressed by control preys might physically interact with any membrane protein, particularly itself, has remained unsolved. In order to clarify this issue, we developed the novel set of control preys (pDL2-AG5HR2 and pAI-AG5HR2) and examined their properties as experimental controls. In conclusion, pDL2-AG5HR2 construct expressed Alg5HR2p-NubG, which did not interact with an Alg5p portion of Alg5p-Cub bait protein, while pAI-AG5HR2 construct expressed Alg5HR2p-NubI, which did interact with a Cub portion of Alg5p-Cub bait protein via its NubI portion. These results show that the novel set of control preys were more suitable than conventional set of those for analysis of physical interaction regarding at least Alg5p. Although we presently cannot exclude the possibility that the Alg5HR2p portion might interact with unknown protein, our further research will prove availability of these preys.

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