A Novel Indication of the Activities of Small Molecule Inhibitors of Protein-protein Interactions: Application of the Yeast Two-hybrid System

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Numerous cellular processes, such as signal transduction and protein transport, are regulated through protein-protein interactions[1]. To reveal the roles of these interactions *in vivo*, it is important to investigate the effect of inhibition of each protein-protein interaction. Small molecule inhibitors of protein-protein interactions are useful for such purposes, but only a few inhibitors have been identified[2,3]. The lack of effective screening methods may be a main reason for this situation.

The possible application of the yeast two-hybrid system for identifying small molecule inhibitors of protein-protein interactions was suggested by FIELDS and STERNGLANZ[4]. In the original yeast two-hybrid system, protein-protein interaction in yeast leads to the expression of a reporter gene *HIS3*, which permits the yeast to grow without histidine. Therefore, small molecule inhibitors of the protein-protein interactions can be selected as growth inhibitors of the yeast in the medium without histidine. However, this principle is impractical itself, because growth inhibitors, such as protein synthesis inhibitors, DNA synthesis inhibitors and respiration inhibitors, would also be selected in the actual screening and are difficult to exclude.

We predicted that the true inhibitors of protein-protein interactions should inhibit the growth of the yeast in the medium without histidine but not in that with histidine. Therefore, we defined ΔI%, a difference between the growth inhibition % without histidine and with histidine, as a new indication of the protein-protein interaction inhibitory activity of test compounds. The feasibility of ΔI% was confirmed by evaluating the activity of ascomycin[5], an FK506-derivative, which binds FK506 binding protein (FKBP) and thereby inhibits the interaction of type I transforming growth factor beta (TGF-β) receptors with FKBP. Using ΔI% as an indication, the original yeast two-hybrid system can be applied as a simple method for selecting small molecule inhibitors of protein-protein interactions.

Materials and Methods

Yeast Two-hybrid System

The original yeast two-hybrid system was purchased from Clontech. The cDNAs of ALK5 and FKBP[6] were cloned by PCR using human leukocyte cDNA library as a template and their sequences were confirmed by DNA sequencing. The cytoplasmic domain of ALK5 was PCR amplified and subcloned into pGBT9 and used as a bait plasmid. The full length FKBP was PCR amplified and subcloned into pGAD424 and used as a prey plasmid. Primer pairs were: sense 5′-GGAATTCCGACGGAGG-CAGG-3′, antisense 5′-ACGCGTCGACCTATTGA-ATCACTTTAGGCTTCTCTGG-3′ for ALK5. sense 5′-GGAATTCATGGGAGTGCAGGTGGAA-3′, antisense 5′-CCGCTCGAGTTATCATTCCAGTTTTA-GAAGCTCC-3′ for FKBP.

Assay Procedure

Yeast reporter strain PJ69-2A (*MATa*, *ura3-52*, *his3-200*, *trp1-901*, *leu2-3*, *112*, *gal4Δ*, *gal80Δ*, *LYS2*::$GAL1_{UAS}$-$GAL1_{TATA}$-*HIS3*, $GAL2_{UAS}$-$GAL2_{TATA}$-*ADE2*) was transformed with designated plasmid. The resulted transformants were seeded in 2 ml SD/-LW medium (yeast nitrogen base 0.67%, glucose 2%, various nutrients without leucine and tryptophan) and incubated at 30°C overnight. After washing twice with SD/-LWH (yeast nitrogen base 0.67%, glucose 2%, various nutrients without leucine, tryptophan and histidine), the cells were diluted to optical density of 0.1 with SD/-LW or SD/-LWH and 200 μl of the diluted cultures were put into the wells of 96 well culture plates. Various concentrations of ascomycin were added to the wells and the plates were incubated at 30°C for 72 hours. The optical density at 600 nm of the culture was read with the microplate reader and ΔI% of each concentration of ascomycin was calculated as indicated in Results and Discussion.

Results and Discussion

We defined $\Delta I\%$, a new indication of the protein-protein interaction inhibitory activity of sample compounds, as an equation noted below.

$$\Delta I\% = I\%(-H) - I\%(+H)$$

where:

$$I\% = (OD(-sample) - OD(+sample)) / OD(-sample) \times 100$$

OD = optical density at 600 nm

−H = medium without histidine

+H = medium with histidine

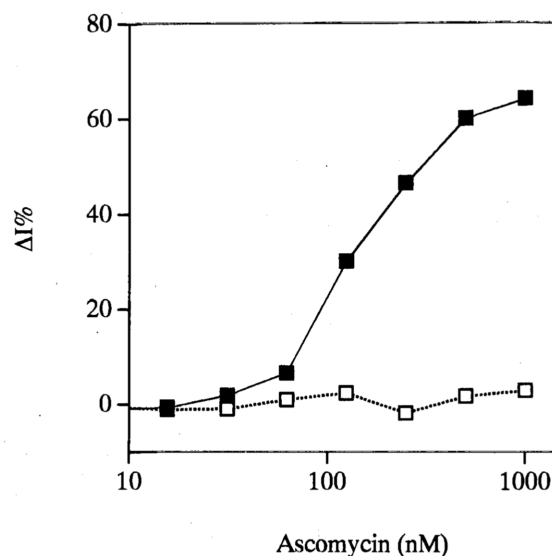
$\Delta I\%$ represents the inhibitory activity of each sample on *HIS3*-dependent growth of the yeast and consequently, it should represent the protein-protein interaction inhibitory activity.

We have tested whether $\Delta I\%$ is a useful indication by evaluating the inhibitory activity of ascomycin, an FK506-derivative, to the interaction of ALK5 with FKBP. The cytoplasmic region of ALK5 was fused to the DNA binding domain of Gal4 and the full length FKBP was fused to the transcriptional activating domain of Gal4. The resulted plasmids were co-transformed into the yeast PJ69-2A. As a control, the plasmid for expressing the full length Gal4 was transformed into the same strain. First, the histidine-less growth of the transformants was confirmed. Optical density of the yeasts rose in incubation time-dependent manner (data not shown). Next, the activity of ascomycin was quantified using $\Delta I\%$ as an indication. Various concentrations of ascomycin were added to the yeast culture and incubated for 72 hours at 30°C. Optical density of the culture was measured with a 96 well microplate reader. $\Delta I\%$ was calculated and plotted to the concentration of ascomycin. As shown in Fig. 1A, $\Delta I\%$ rose in dose-dependent manner at maximum of 64% when the yeast co-transformed with ALK5 and FKBP was used. The half maximal effective concentration of ascomycin was 200 nM. On the other hand, $\Delta I\%$ was almost none when the yeast transformed with Gal4 was used. In addition, as shown in Fig. 1B, $\Delta I\%$ at 500 nM ascomycin rose in time-dependent manner when the yeast co-transformed with ALK5 and FKBP was used. We next tested cycloheximide, a protein synthesis inhibitor to show that this assay system does not select general growth inhibitors. Fig. 2 shows $\Delta I\%$ of cycloheximide was almost none with both yeasts and indicates the general growth in-

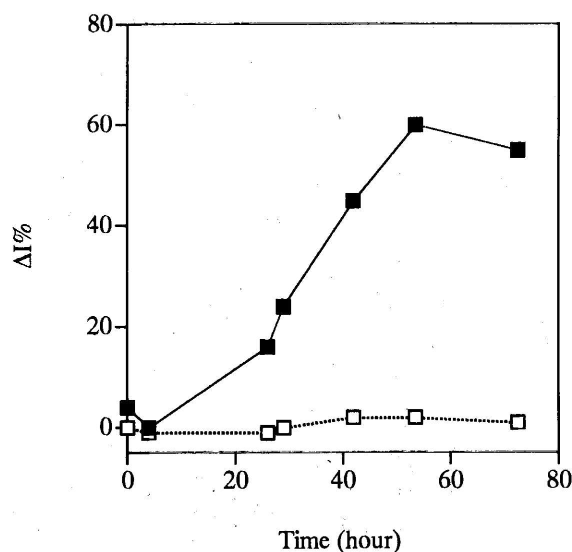
Fig. 1. Inhibition of interaction between ALK5 and FKBP by ascomycin.

The cultures of yeast PJ69-2A transformed with Gal4 DB-ALK5 and Gal4 TA-FKBP (■) or control yeast PJ69-2A transformed with Gal4 full length (□) were suspended in the medium with histidine or without histidine and transferred into wells of 96-well plates.

(A) Various concentrations of ascomycin were added to the wells and incubated for 72 hours at 30°C.



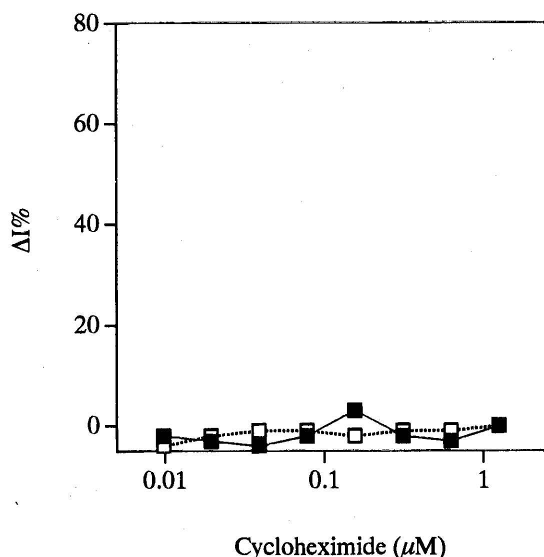
(B) 500 nM ascomycin was added to the wells and incubated for indicated periods at 30°C.



Optical density at 600 nm of the wells was read with microplate reader. $\Delta I\%$ of each concentration of ascomycin was plotted.

Fig. 2. Effect of cycloheximide on ΔI%.

The cultures of yeast PJ69-2A transformed with Gal4 DB-ALK5 and Gal4 TA-FKBP (■) or control yeast PJ69-2A transformed with Gal4 full length (□) were suspended in the medium with histidine or without histidine and transferred into wells of 96-well plates.



Various concentrations of cycloheximide were added to the wells and incubated for 72 hours at 30°C. Optical density at 600 nm of the wells was read with microplate reader. ΔI% of each concentration of cycloheximide was plotted.

hibitors, such as cycloheximide, may inhibit the growth of the yeast with or without histidine and therefore results in low ΔI% value. Taken together, we conclude that the inhibitory activity of protein-protein interactions of test samples can be semi-quantified simply with the yeast two-hybrid system using ΔI% as an indication.

In the actual screening using ΔI%, the Gal4 function inhibitors and the His3 activity inhibitors would also be selected. These false positives would be excluded as those that inhibit the histidine-minus growth of the yeast transformed with Gal4. The true positives, such as ascomycin, should have considerably high ΔI% with the yeast expressing the target proteins but low ΔI% with the yeast expressing Gal4 (Fig. 1A).

Recently, the inducible reverse two-hybrid assay for selecting small molecule inhibitors of protein-protein interactions was reported[7]. In that assay, the protein-protein interaction in yeast leads to the expression of a reporter gene *URA3*, which converts the 5-fluoroorotic acid (5-FOA) to a toxic substance. Therefore, small molecules with protein-protein interaction inhibitory activities would be selected as those which recover the growth of the yeast in 5-FOA-containing media. The growth inhibitors would inhibit the growth of the yeast and therefore be excluded. The feasibility of that assay system has been shown in that FK506 recovers the growth of the yeast expressing TGF beta receptor I and FKBP in 5-FOA-containing media. That system is excellent in that the true positive samples will be selected as those which recover the growth of the yeast. However, in the case of mass screening, we point out the two unfavorable properties, *i.e.* cost and rather complex operation. That system requires 5-FOA, a comparatively expensive material in assay media. In addition, it requires the change of sugar material from glucose to galactose in order to express the target proteins. On the other hand, the original two-hybrid system used here require no extra materials or the change of sugar.

Using ΔI% as an indication of inhibitory activities of test samples, we have shown that the original two-hybrid system is applicable to screen the small molecule inhibitors of protein-protein interactions. The selected inhibitors would be useful therapeutic agents as well as molecular tools for studying the roles of each protein *in vivo*.

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