Figure 4. Histogram of the affinity force between aptamer and thrombin for 4096 data points. (A) TBA-1, (B) TBA-2, (C) conventional thrombin aptamer and (D) anti-thrombin antibody.

Table 2. The average of affinity force between obtained aptamer and thrombin

	Average of affinity force (pN) (mean ± SE)	Peak position (pN)
N60	57.15 ± 0.96	10–19
TBA-1	205.52 ± 0.70	210–219
TBA-2	74.48 ± 0.90	40–49, 110–119
Conventional thrombin aptamer	65.09 ± 0.98	10–19
Anti-thrombin antibody	91.59 ± 1.09	50–59

thrombin could be measured. Furthermore, the SELEX cycle number was suitable with three round since the affinity of TBA-1 to thrombin is significantly larger than that of anti-thrombin antibody at three rounds.

Sequence analysis

The third-round elution DNA that suggested the strongest binding to thrombin was subcloned to pT7 blue vector, and the sequence was determined. Many of the resultant obtained DNA sequences had a G-rich sequence (Table 1). Previously, thrombin aptamer possessing a G-rich sequence had been reported (14). According to this previous reported result, thus, it was assumed that the oligonucleotide sequence called TBA-1 could be functioned as a DNA aptamer against with thrombin.

Binding assay and determination of dissociation constant

The histogram of the affinity force between obtained aptamers called TBA-1 and -2 and thrombin was depicted from 4096 points of force curves (Figure 4A
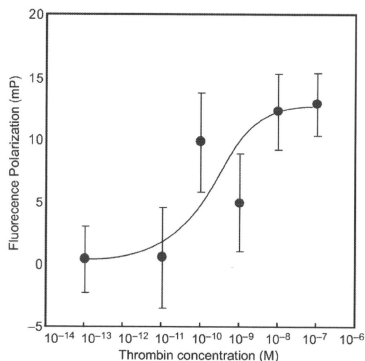
Figure 5. Binding curve of TBA-1 with thrombin. Each of thrombin solution (10^{-7}–10^{-13} M) was mixed with TBA-1 (100 nM), and incubated for 2 h at room temperature. After incubation, the polarization was measured.

Table 3. The fluorescence polarization value between TBA-1 and thrombin or streptavidin

	Polarization value (mP)	SE
Thrombin	12.9	2.60
Avidin	0.23	3.25

and B). The force histogram of TBA-1 had one peak at 210–219 pN (Figure 4A). These results suggest that there is a single binding site of TBA-1 to thrombin. On the other hand, the histogram of TBA-2 has three peaks at 40–49 and 110–119 pN (Figure 4B). The force value of the second peak is twice as large as that of the first peak. For these reasons, TBA-2 is considered to have two binding sites to thrombin.

The average affinity force between aptamer and thrombin was calculated from 4096 point force curve data (Table 2). The force average of TBA-1 was 203.52 pN, and the affinity of the N60 pool to thrombin was 57.14 pN. These results indicate that obtained ssDNAs have a high affinity to thrombin.

On the other hand, the conventional thrombin aptamers that have the 'GGTTGGTGTGGTTGG' sequence have the affinity force of 65.09 pN (Figure 4C and Table 2). Therefore, the affinity of obtained DNA aptamers to thrombin is stronger than that of the conventional thrombin aptamer. In previously report, the average of affinity force between conventional selected thrombin aptamer and thrombin is 4.45 pN. However, in this study, the affinity force was estimated to be 65.09 pN. It was considered that this difference was depended on the immobilization method and the DNA aptamer molecule number involved in immobilization. In addition, the

affinity force between anti-thrombin antibody and thrombin is 91.59 pN (Figure 4D and Table 2). Therefore, the affinity of the TBA-1 aptamer to thrombin is stronger than that of antibody to thrombin.

For determination of dissociation constant (K_D), the fluorescence polarization analysis was carried out. As a result, binding curve was increased with thrombin concentration dependency (Figure 5). However, in the case of streptavidin, the polarization value of FITC-labeled TBA-1 was not increased significantly (Table 3). Therefore, it is considered that the binding affinity of TBA to thrombin is specifically. By fluorescence polarization analysis, the K_D value of TBA-1 against thrombin is estimated to be 200 pM (2×10^{-10} M). Although K_D value of streptavidin–biotin interaction is 1×10^{-14} order, however, as a result, the binding affinity of TBA-1 to thrombin is 10 000-fold lower than that of streptavisdin–biotin interaction. Generally, free streptavidin exhibited a high affinity interaction described above. However, in the case of immobilized streptavidin, the K_D value to biotin was decreased to 1×10^{-8} to 1×10^{-10} M order by conformation alternation (30). In AFM SELEX analysis, the streptavidin was covalently immobilized on the tip of surface of cantilever. Therefore, it was considered that K_D value of streptavidin was decreased by immobilization, and as a result, the DNA aptamers with comparable lower affinity were selected.

As mentioned above, the K_D value of isolated DNA aptamer TBA-1 was 1000 times lower than conventional aptamer having 200 nM of dissociation constant to thrombin (14). Moreover, this result was strongly supported to AFM analysis of TBA-1 affinity. Moreover, by AFM analysis, there was significant difference between the affinity of TBA-1 and the affinity of antibody to thrombin, and affinity force of TBA-1 was strongly compared with that of anti-thrombin antibody. Therefore, it was considered that the dissociation constant of TBA-1 to thrombin is $<10^{-8} - 10^{-10}$ M, since the dissociation constant of antibody to antigen is $\sim 10^{-8} - 10^{-10}$ M (23–27). However, there was no significant difference of dissociation constant between TBA-1 and antibody to thrombin. Therefore, it was considered that TBA-1 was optimized on AFM analysis.

In many reports, the dissociation constant of aptamers to their target molecules is 10 – 0.01 μM, and the aptamers that have low affinity are also selected (7–17). This is because the affinity force of obtained aptamers to their target cannot be controlled when using a conventional SELEX method. This AFM-SELEX strategy permit us to not only selecting quickly, but also obtaining high affinity DNA aptamer to the target as well as that of antibody to antigen. Therefore, this method may have a practical advantage for obtaining aptamers having a strong affinity to targets.

Concluding remarks

In this study, a DNA aptamer that binds to thrombin with very high affinity and specificity was selected using AFM. The affinity force between ssDNA and thrombin grew gradually stronger upon repeating selection rounds.

In addition, the sequences of obtained aptamers have many G-rich regions. One type of obtained aptamers, called TBA-1, has a strong affinity to thrombin compared with the conventional thrombin aptamer. This result suggests that DNA aptamers that bind to their targets with high affinity can be selected by AFM-SELEX. In addition, it suggests that DNA aptamers could be selected using fewer rounds, as compared with a conventional SELEX strategy. Considered together, these results indicate that this new SELEX strategy could be a viable candidate for the screening for various DNA aptamers with high affinities.

FUNDING

Conflict of interest statement. None declared.

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The competitor-introduced Gγ recruitment system, a new approach for screening affinity-enhanced proteins

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We have developed a new approach based on the Gγ recruitment system to screen affinity-enhanced proteins by expressing a binding competitor. The previously established Gγ recruitment system is a yeast two-hybrid (Y2H) system that utilizes G-protein signaling, and is based on the fact that membrane localization of the G-protein γ subunit (Gγ) is essential for signal transduction in yeast. In the original Y2H system, an engineered Gγ that lacks membrane localization upon deletion of the lipid modification site (Gγ$_{cyto}$) is produced, and a candidate protein with an artificial lipidation site and its counterpart fused with Gγ$_{cyto}$ are expressed. As protein–protein interactions bring Gγ$_{cyto}$ towards the plasma membrane, G-protein signaling can be activated, and the interaction is detected by various cellular responses as the readout. In the current study, we expressed a third cytosolic protein that competes with the candidate protein to specifically isolate affinity-enhanced mutants from a mutation library of the candidate protein. Enhancing the affinity of the protein candidate guides the counterpart–Gγ$_{cyto}$ fusion protein towards the plasma membrane and activates signaling. Using mutants of the Z domain derived from *Staphylococcus aureus* protein A as candidate proteins or competitors, and the Fc portion of human immunoglobulin G (IgG) as the counterpart, we demonstrate that affinity-enhanced proteins can be effectively screened from a library containing a 10 000-fold excess of non-enhanced proteins. This new approach, called the competitor-introduced Gγ recruitment system, will be useful for efficient discovery of rare valuable candidates hidden among excess ordinary ones.

Structured digital abstract
- MINT-7556266: Fc portion of human IgG (uniprotkb:P01857) physically interacts (MI:0915) with Z domain of protein A (uniprotkb:P38507) by two hybrid (MI:0018)

Introduction

Directed evolution is an extremely useful approach in protein engineering that is used to produce novel proteins with desirable properties that are not found in nature [1–3]. This approach has been successfully applied to engineer a wide range of protein functions, such as activity, stability, selectivity, specificity and affinity [4]. 'Bio-panning' is broadly used for the engineering of protein affinity, mostly based on phage

display techniques [5]. This approach makes it possible to isolate affinity-enhanced variants from a library under highly specific elution conditions; however, it is difficult to design suitable elution conditions, and the procedure may require multiple cycles of isolation and amplification to exclude non-enhanced variants.

Recently, the use of yeast two-hybrid (Y2H) systems for affinity enhancement has been reported [3,6]. These systems successfully enhance the affinity of target proteins towards their binding partners by regulating the concentration of the partners. A low concentration leads to a reduction in sensitivity such that the interaction cannot be detected; therefore, only affinity-enhanced variants can be isolated in these systems. Unfortunately, these applications are limited to particular interactions such as receptor–ligand interactions or interactions in nature that are originally weak.

Here we propose a new approach based on a Y2H system to enhance protein affinity by expressing a binding competitor. The competitor-introduced Y2H system can specifically isolate affinity-enhanced variants from a genetically mutated library by expressing the original or an improved protein as a competitor. The advantage of this approach is that it can be easily used for screening binding partners with quite strong affinities and various candidates just by altering the competitor, and requires just a single cycle of isolation. In this study, we utilized the Gγ recruitment system, a Y2H system that utilizes yeast G-protein signaling [7], to demonstrate the applicability of the competitor-introduced approach for affinity enhancement. The Z domain derived from staphylococcal protein A was selected as a model protein for affinity enhancement, and the Fc portion of human IgG was selected as its counterpart [8,9].

Results

Competitor-introduced Gγ recruitment system

The Gγ recruitment system is a Y2H system that was previously designed to detect protein–protein interactions based on the finding that signal transduction requires localization of the Gβγ complex to the plasma membrane through a lipidated Gγ subunit in yeast [10]. Formation of Gγ mutants by deletion of their lipidation sites completely interrupts G-protein signaling [10], and protein–protein interactions lead to activation of G-protein signaling by recruiting the Gγ mutants towards the plasma membrane [7]. The outputs appear as various cellular responses, including global changes in transcription in preparation for mating.

An outline of our strategy for affinity enhancement, designated the competitor-introduced Gγ recruitment system, is shown in Fig. 1. The expression of binding competitor 'C' in the cytosol (C_{cyto}) affects the interaction between target protein 'A', which is genetically fused to a cytosolic Gγ mutant ($G\gamma_{cyto}$), and binding candidate 'B', which is artificially anchored at the plasma membrane (B_{mem}). When the affinity between 'A' and 'B' is lower than that between 'A' and 'C', the 'A'–$G\gamma_{cyto}$ fusion protein preferentially binds to C_{cyto} and cannot localize to the plasma membrane, and therefore the G-protein signal is not activated (Fig. 1A). In contrast, when 'B' binds to 'A' more strongly than to 'C', the 'A'–$G\gamma_{cyto}$ fusion protein migrates towards the B_{mem} protein at the plasma membrane, the G-protein signal is activated, and the cellular response in the yeast mating process is induced (Fig. 1B).

To verify the efficacy of the strategy described in Fig. 1, we examined whether affinity-enhanced variants specifically induce signal transduction in haploid cells
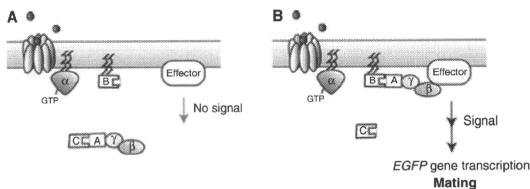

Fig. 1. Outline of the experimental design. Engineered Gγ lacking membrane localization ability ($G\gamma_{cyto}$) is genetically prepared, and binding target 'A' is fused to $G\gamma_{cyto}$. Binding candidate 'B' is located on the plasma membrane and the competitor 'C' is introduced into the cytosol. (A) When 'A' prefers to bind to 'C', G-protein signaling is prevented by sequestration of $G\gamma_{cyto}$ from the plasma membrane. (B) When 'A' prefers to bind to 'B', G-protein signaling is transmitted to induce *EGFP* gene transcription and the yeast mating process.

in the presence of the competitors, and whether the resulting signal can be used to screen affinity-enhanced variants using diploid cell formation.

Verification of the growth selection method using the mating machinery to screen protein–protein interactions in the Gγ recruitment system

Yeast haploid strains BY4741, consisting of a specific methionine prototrophic **a** cell, and BY4742, consisting of a specific lysine prototrophic α cell [11], were utilized as parental strains for construction of our system. The genetic modifications shown in Table 1 were performed for BY4741 only, and the recovery of phero-mone signaling in the engineered **a** cell was used to detect protein–protein interactions. Briefly, the interaction between 'A'–Gγ$_{cyto}$ and 'B$_{mem}$' restores signaling and induces transcription of the *EGFP* reporter gene in the Gγ recruitment system as described previously [7], in addition to simultaneously activating the cellular responses required for the mating process. To test the screening procedure, we examined whether the yeast mating machinery can be used to screen signaling-recovered cells by protein–protein interactions as in our previous system. The engineered **a** cell that restores pheromone signaling by protein–protein interactions mates with an intact α cell, and the diploid cell generated survives on medium lacking methionine and lysine.

Interactions of BFG2Z18-K35A, BFG2Z18-WT and BZFG2118 (Tables 1 and 2), which express the Fc portion of human IgG as protein 'A', with several Z variants with various affinities for the Fc portion (Z$_{K35A}$, 4.6×10^6 M^{-1}; Z$_{WT}$, 5.9×10^7 M^{-1}; ZZ, 6.8×10^8 M^{-1}) [12] as protein 'B', transduce pheromone signaling [7]. However, BFG2118 (Tables 1 and 2), which

Table 2. List of proteins expressed in the engineered yeast strains.

Strain	Membrane target protein (A)	Gγ$_{cyto}$ fusion protein (B)	Competitor protein (C)
BFG2118	–	Gγ$_{cyto}$-Fc	–
BFG2Z18-K35A	Z$_{K35A,mem}$	Gγ$_{cyto}$-Fc	–
BFG2Z18-WT	Z$_{WT,mem}$	Gγ$_{cyto}$-Fc	–
BZFG2118	ZZ$_{mem}$	Gγ$_{cyto}$-Fc	–
FC1-1	Z$_{K35A,mem}$	Gγ$_{cyto}$-Fc	Z$_{K35A}$
FC2-1	Z$_{WT,mem}$	Gγ$_{cyto}$-Fc	Z$_{K35A}$
FC3-1	ZZ$_{mem}$	Gγ$_{cyto}$-Fc	Z$_{K35A}$
FC1-2	Z$_{K35A,mem}$	Gγ$_{cyto}$-Fc	Z$_{WT}$
FC2-2	Z$_{WT,mem}$	Gγ$_{cyto}$-Fc	Z$_{WT}$
FC3-2	ZZ$_{mem}$	Gγ$_{cyto}$-Fc	Z$_{WT}$

is a negative control and expresses the Fc protein fused to the Gγ$_{cyto}$ protein, cannot trigger signal transduction [7]. To verify the feasibility of growth selection via the yeast mating machinery, these four strains were co-cultivated with intact mating partner BY4742 (Table 1) and then spotted onto diploid selectable methionine- and lysine-lacking medium. As a result, BFG2118 did not survive but the other three strains were able to grow (Fig. 2A). To quantitatively estimate the survival of these strains, 1 mL of cell suspension from each strain (attenuance at 600 nm adjusted to 1.0; $D_{600} = 1.0$) was spread on the same selection medium, and the colony numbers were counted. There were obvious differences in colony numbers, corresponding to the affinity constants shown in Fig. 2B. These results suggest that the mating abilities of the **a** cells were retrieved and diploid cells were produced in agreement with signaling in response to protein–protein interactions, and that the growth selection method using yeast mating is adequate to screen candidates for protein–protein interactions in the Gγ recruitment system.

Table 1. List of yeast strains used in this study.

Strain	Genotype	Reference sources
BY4741	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	Brachmann *et al.* (1998)
MC-F1	BY4741 *P$_{FIG\ 1}$-FIG 1-EGFP*	Ishii *et al.* (in preparation)[a]
BFG2118	MC-F1 *ste18Δ::kanMX4 his3Δ::URA3-P$_{STE18}$-Gγ$_{cyto}$-Fc*	Fukuda *et al.* (2009)
BFG2Z18-K35A	MC-F1 *ste18Δ::kanMX4-P$_{PGK1}$-Z$_{K35A,\ mem}$ his3Δ::URA3-P$_{STE18}$-Gγ$_{cyto}$-Fc*	Fukuda *et al.* (2009)
BFG2Z18-WT	MC-F1 *ste18Δ::kanMX4-P$_{PGK1}$-Z$_{WT,\ mem}$ his3Δ::URA3-P$_{STE18}$-Gγ$_{cyto}$-Fc*	Fukuda *et al.* (2009)
BZFG2118	MC-F1 *ste18Δ::kanMX4-P$_{PGK1}$-ZZ$_{mem}$ his3Δ::URA3-P$_{STE18}$-Gγ$_{cyto}$-Fc*	Fukuda *et al.* (2009)
FC1-1	BFG2Z18-K35A *P$_{HOP2}$::LEU2-P$_{PGK1}$-Z$_{K35A}$*	Present study
FC2-1	BFG2Z18-WT *P$_{HOP2}$::LEU2-P$_{PGK1}$-Z$_{K35A}$*	Present study
FC3-1	BZFG2118 *P$_{HOP2}$::LEU2-P$_{PGK1}$-Z$_{K35A}$*	Present study
FC1-2	BFG2Z18-K35A *P$_{HOP2}$::LEU2-P$_{PGK1}$-Z$_{WT}$*	Present study
FC2-2	BFG2Z18-WT *P$_{HOP2}$::LEU2-P$_{PGK1}$-Z$_{WT}$*	Present study
FC3-2	BZFG2118 *P$_{HOP2}$::LEU2-P$_{PGK1}$-Z$_{WT}$*	Present study
BY4742	*MATα his3Δ1 ura3Δ0 leu2Δ0 lys2Δ0*	Brachmann *et al.* (1998)

[a] J. Ishii, M. Moriguchi, S. Matsumura, K. Tatematsu, S. Kuroda, T. Tanaka, T. Fujiwara, H. Fukuda & A. Kondo, unpublished results.
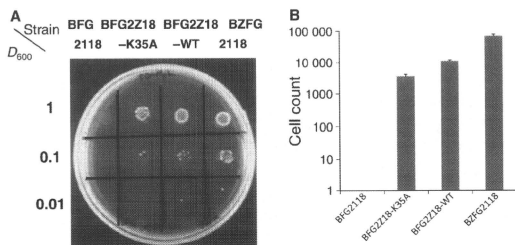
Fig. 2. Restoration of mating ability by protein–protein interactions without competitors. (A) Growth assay to test the mating ability of yeast strains. (B) Quantitative evaluation of mating ability indicated by the number of diploid cells formed by 1 mL of cell suspension with D_{600} set at 1.0. BY4742 was used as the mating partner. Standard errors of three independent experiments are shown.
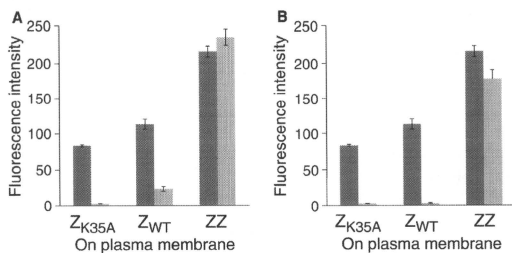

Fig. 3. Flow cytometric EGFP fluorescence analyses for comparing the G-protein signal level. (A) Fluorescence intensity measured in the competitor Z_{K35A}-introduced strains (yeast strain generated by introducing Z_{K35A} as the competitor) (FC1-1, FC2-1 and FC3-1). (B) Fluorescence intensity measured in the competitor Z_{WT}-introduced strains (yeast strain generated by introducing Z_{WT} as the competitor) (FC1-2, FC2-2 and FC3-2). Dark gray bars indicate yeast strains without competitors (BFG2Z18-K35A, BFG2Z18-WT and BZFG2118), and light gray bars indicate competitor-introduced strains. To investigate transduction of the signal, 5 μM of α factor was used for each strain. Standard errors of three independent experiments are shown.

Expression of an interacting competitor inhibits the restoration of signaling in the Gγ recruitment system and excludes the detection of non-enhanced variants

To examine whether the expression of competitors prevents the recovery of G-protein signal transduction as shown in Fig. 1, two competitors, soluble Z_{K35A} and Z_{WT}, were introduced singly into three **a**-type strains, BFG2Z18-K35A, BFG2Z18-WT and BZFG2118 (Tables 1 and 2), and signal transduction was quantitatively evaluated based on transcriptional activity of the *EGFP* reporter gene. Fig. 3A shows the results for yeast strains with Z_{K35A} as the competitor. FC1-1 (Gγ$_{cyto}$–Fc/$Z_{K35A,mem}$/Z_{K35A}) exhibited no fluorescence and a significant decrease in fluorescence intensity occurred in

FC2-1 (Gγ$_{cyto}$–Fc/$Z_{WT,mem}$/Z_{K35A}) upon expression of the competitors, but no decay of fluorescence was observed in FC3-1 (Gγ$_{cyto}$–Fc/ZZ_{mem}/Z_{K35A}). In the case of yeast strains possessing Z_{WT} as the competitor (Fig. 3B), FC1-2 (Gγ$_{cyto}$–Fc/$Z_{K35A,mem}$/Z_{WT}) and FC2-2 (Gγ$_{cyto}$–Fc/$Z_{WT,mem}$/Z_{WT}) did not exhibit fluorescence, and a slight decrease in fluorescence intensity occurred in FC3-2 (Gγ$_{cyto}$–Fc/ZZ_{mem}/Z_{WT}). These results suggest that expression of competitors in the cytosol strongly affected signal transduction by inhibiting the interactions between Gγ$_{cyto}$-fused Fc and several partners attached to the plasma membrane, and completely interrupted the migration of Gγ$_{cyto}$ towards the plasma membrane when the affinity constant of the competitor was equal to or greater than that of the membrane-associated binding partner.
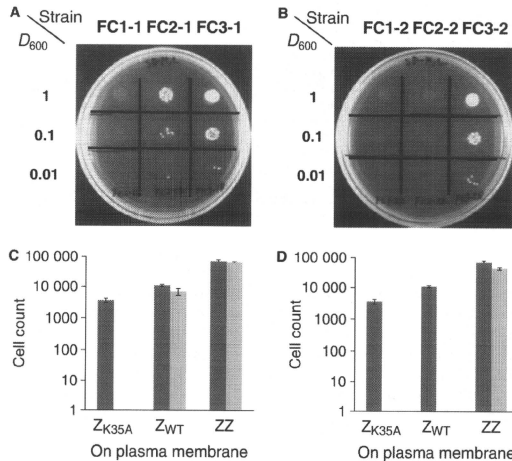
Fig. 4. Evaluation of the mating ability of competitor-introduced strains. (A) Growth assay to test the mating ability of yeast strains possessing competitor Z_{K35A}. (B) Growth assay to test the mating ability of yeast strains possessing competitor Z_{WT}. (C) Quantitative evaluation of the mating ability of yeast strains possessing competitor Z_{K35A}. (D) Quantitative evaluation of the mating abilities of yeast strains possessing competitor Z_{WT}. Dark gray bars indicate yeast strains without competitors (BFG2Z18-K35A, BFG2Z18-WT and BZFG2118), and light gray bars indicate competitor-introduced strains. Mating ability was quantitatively evaluated by the number of diploid cells formed by 1 mL of cell suspension, with D_{600} set at 1.0. BY4742 was used as the mating partner. Standard errors of three independent experiments are shown.

Use of the competitor-introduced Gγ recruitment system to screen affinity-enhanced variants

To verify the ability of the competitor expression to screen affinity-enhanced variants, the mating abilities of yeast strains possessing competitors were evaluated (Fig. 4). In agreement with the *EGFP* reporter assay, FC1-1 did not generated diploid cells, while FC2-1 and FC3-1 grew on the diploid selectable medium (Fig. 4A). The mating abilities of FC2-1 and FC3-1 were almost equivalent to those of yeast strains without Z_{K35A} as the competitor (Fig. 4C). Similar results were obtained in the case of yeast strains possessing Z_{WT} as the competitor. FC1-2 and FC2-2 did not exhibited mating ability; only FC3-2 generated diploid cells and exhibited an almost equivalent mating ability to yeast strains without the competitor (Fig. 4B,D). These results suggest that introduction of an appropriate competitor is adequate for screening superior variants of binding partners compared to original partners.

Finally, to clarify the capabilities of the competitor-introduced Gγ recruitment system for affinity enhance-

ment, screening efficiencies were evaluated using model libraries as follows. The Z domain and ZZ domain were selected as model proteins of an original binding partner and affinity-enhanced binding partner, respectively, and two artificial libraries were prepared. One contained a minor amount of BZFG2118 as the affinity-enhanced target mutant and an excess amount of BFG2Z18-WT as the original affinity molecule, while the other contained a minor amount of FC3-2 as the affinity-enhanced target mutant and an excess amount of FC2-2 as the original affinity molecule. Several mixing ratios were used as shown in Table 3. The screening efficiency was defined as the ratio of target cells that were obtained on the selection plate divided by the initial ratio of target cells. These values were assessed by observing the difference in fragment sizes between the Z domain (as the original molecule) and the ZZ domain (as the target mutant) using PCR. As shown in Table 3, the screening efficiency using competitor-introduced system was much greater than that using a conventional system without a competitor, and the maximum screening efficiency reached 7000-fold.

Table 3. Screening efficiency of target cells from model libraries using growth selection via yeast mating.

Competitor-introduced system consisting of FC3-2 and excess FC2-2			Previous system consisting of BZFG2118 and excess BFG2Z18-WT		
Initial ratio of target cells (%)	Final ratio of target cells (%)	Screening efficiency	Initial ratio of target cells (%)	Final ratio of target cells (%)	Screening efficiency
10	100	10	10	60	6
1	100	100	1	0	0
0.1	100	1000	–	–	–
0.01	70	7000	–	–	–

Discussion

The aim of this study was to establish a novel approach for affinity enhancement that can be applied to a diverse range of proteins on the basis of the Y2H system. The Z domain derived from *Staphylococcus aureus* protein A and the Fc portion of human IgG, which are widely used as a model interaction pair, were used to demonstrate the feasibility of our system [13–16]. The Z domain has a number of variants with a wide range of affinity constants to the Fc portion, such as Z_{K35A} (4.6×10^6 M^{-1}), Z_{WT} (5.9×10^7 M^{-1}) and ZZ (6.8×10^8 M^{-1}) [12], which makes them useful for verifying our new affinity enhancement strategy.

In our system, protein–protein interactions were converted into G-protein signals through localization of the yeast Gγ subunit to the plasma membrane, and detected by fluorescence intensity using transcriptional activation of an *EGFP* reporter gene in response to signal transduction [7]. Although use of a fluorescence reporter allows quantitative assessment of the change in the signaling level and high-throughput screening [17], it requires access to a flow cytometer [18]. As a simpler isolation technique to detect positive clones without any expensive instruments, we verified the adequacy of growth selection by diploid formation based on the yeast mating machinery in the current study.

First, we investigated whether the mating machinery can detect the restoration of pheromone signaling due to protein–protein interactions using our previous Gγ recruitment system [7]. The results of cell growth on diploid selectable medium clearly demonstrated the efficacy of growth selection to screen for protein–protein interaction pairs with affinity constants ranging from 4.6×10^6 to 6.8×10^8 M^{-1} (Fig. 2). Although we successfully detected the interaction between Z_{131K} and Fc (8.0×10^3 M^{-1}) by transcriptional assay of an *EGFP* reporter gene in a previous study, we did not prepare and test variants with marginal affinity in the present study because it focuses on affinity enhancement for protein engineering. The complete elimination

of background growth with a non-interacting pair (BFG2118) (Fig. 2) clearly shows the usefulness of the mating machinery for screening with our previous system. This extremely low background is due to the fact that retrieval of signaling is strictly regulated by protein–protein interactions, and formation of the diploid absolutely requires the recovered signaling. Although our previous system is able to discriminate interacting pairs from non-interacting pairs, it is not sufficient for screening for affinity-enhanced variants from a pool of original interacting pairs, suggesting that another approach is required for efficient screening of affinity enhancement (Table 3).

As shown in Fig. 1, we hypothesized that expression of a cytosolic competitor for a membrane-associated protein could restrict signaling transduction as the competitor might intercept the Gγ-fused protein and interrupt its migration towards the plasma membrane. Indeed, introduction of competitors eliminated the interactions of relatively weaker binders, while superior binders on the plasma membrane easily transduced the signal even in the presence of competitors (Figs 3 and 4). Furthermore, the competitors completely interrupted migration of the Gγ-fused protein toward the plasma membrane when binders were the same protein as the competitors (Figs 3 and 4), although we utilized the same promoter for expression of the binders and competitors as shown in Table 1. The amount of cytosolic proteins that function as competitors may have exceeded that of the binders that were correctly localized to the plasma membrane. It has been reported that Gγ that genetically lacks either thioacylation or farnesylation fails to localize to the plasma membrane [10], and hence partial leakage of binders into the cytosol might be induced because of the lipid modification process, which we detected using Western blot analyses (data not shown). These results show that our approach enables complete elimination of non-enhanced candidates, and its utility for affinity enhancement of binding partners with quite strong affinities just by altering the competitor.

When soluble Z_{K35A} was expressed as a competitor for membrane-associated Z_{WT} in haploid **a** cells, the G-protein signal observed in the *EGFP* transcription assay was attenuated by competitive inhibition (Fig. 3A), while the mating survival assay showed vigorous diploid formation almost equivalent to that of a yeast strain without a competitor (Fig. 4A). This difference between assays may be due to the fact that mating is triggered by a certain threshold of signaling, while the *EGFP* transcription assay directly reflects the signaling level.

Finally, we quantified screening efficiencies by collecting a small amount of target cells from the model libraries to demonstrate the ability of the competitor-introduced system for affinity enhancement (Table 3). We defined the screening efficiency as the ratio of target cells that were obtained on the selection plate divided by the initial ratio of target cells. Our previous system without competitors displayed a sixfold screening efficiency with 10% of the initial target population, and could not isolate target cells from libraries whose initial target population was < 1%, suggesting that the conventional approach incorrectly selects binders whose affinity constants to target protein are not improved. In contrast, target cells were isolated even from the model library with 0.01% frequency of target cells, and the maximum screening efficiency reached 7000-fold in the competitor-introduced system (Table 3). These results demonstrate the superiority of the competitor-introduced Gγ recruitment system, which can effectively isolate highly affinity-enhanced candidates from a mutational library based on an original binder using just one cycle of isolation.

In conclusion, we established a new approach for enhancing protein affinity based on a Y2H system by expressing a binding competitor. The competitor-introduced Gγ recruitment system can specifically isolate affinity-enhanced variants from libraries containing a large majority of original proteins. This approach can be easily applied to affinity enhancement of various candidates using a single cycle of isolation. Moreover, our competitor-introduced system for affinity enhancement can be applied to other Y2H systems, and may serve as a powerful technical tool for protein engineering.

Experimental procedures

Strains and media

Details of *Saccharomyces cerevisiae* BY4741 [11], BY4742 [11] and other constructed strains used in this study and their genotypes are outlined in Table 1. MC-F1 is a yeast

strain that expresses EGFP under the control of the phero-mone-inducible *FIG 1* promoter (J. Ishii, M. Moriguchi, S. Matsumura, K. Tatematsu, S. Kuroda, T. Tanaka, T. Fujiwara, H. Fukuda & A. Kondo, unpublished results). The yeast strains were grown in YPD medium containing 1% w/v yeast extract, 2% peptone and 2% glucose, or in SD medium containing 0.67% yeast nitrogen base without amino acids (Becton Dickinson, Franklin Lakes, NJ, USA) and 2% glucose. Agar (2% w/v) was added to these media to produce YPD and SD solid media.

Construction of yeast strains

Plasmids used for integration of the Z genes (Z_{WT} and Z_{K35A}) at a position upstream of the *HOP2* gene (P_{HOP2}, *HOP2* promoter region) on the yeast chromosome for subsequent expression in the cytosol as competitors were constructed as follows. The fragments encoding Z variants were amplified from pUMZ-WT and pUMZ-K35A [7] using primers 5'-TTTTGTCGACATGGCGCAACACGA TGAAGCCGTAGACAAC-3' and 5'-AAAAGGATCCTT ATTTCGGCGCCTGAGCAT-3', and inserted into the *SalI*–*Bam*HI sites of pGK425 [19], yielding plasmids pLMZ-WT and pLMZ-K35A, respectively. The fragment used for homologous recombination at the *HOP2* promoter region was amplified from MC-F1 genomic DNA using primers 5'-AAAAGCGGCCGCTTAAAGCAAGGGTAA ATT-3' and 5'-TTTTGAGCTCATCTTTCAAAATAGAGC CTG -3', and inserted into the *Not*I–*Sac*I site of pLMZ -WT and pLMZ-K35A, yielding plasmids pLMZ-WT-H and pLMZ-K35A-H, respectively.

DNA fragments containing each gene were amplified using PCR from plasmids and introduced into the yeast genome using the lithium acetate method [20]. Integration of the Z genes (Z_{WT} and Z_{K35A}) for expression as competitors in the cytosol was achieved by amplifying the DNA fragments containing *LEU2-PGK5'-Z-PGK3'-P*$_{HOP2}$ (*PGK5'*, *PGK1* promoter; *PGK3'*, *PGK1* terminator) from pLMZ-WT-H and pLMZ-K35A-H using 50-nucleotide primers containing a region homologous to that directly upstream of P_{HOP2} (5'-ATACAATTAATTGACATCAGCAGACAGCAAAT GCACTTGATATACGCAGCTCGACTACGTCGTAAG GCCG-3' and 5'-ATCTTTCAAATAGAGCCTGG-3'). The amplified DNA fragments were used to transform BFG2Z18-K35A, BFG2Z18-WT and BZFG2118, and the transformants were selected on SD medium without uracil and leucine, but containing 20 mg·L^{-1} histidine and 30 mg·L^{-1} methionine (SD-Ura,Leu) to yield FC1-1, FC2-1, FC3-1, FC1-2, FC2-2 and FC3-2 strains (Table 1).

Flow cytometric EGFP fluorescence analysis

Fluorescence intensity was measured for Fig 1–EGFP fusion proteins in yeast cells stimulated with 5 μM α-factor in YPD medium at 30 °C for 6 h on a FACSCalibur

(Becton Dickinson) equipped with a 488 nm air-cooled argon laser, and the data were analyzed using CELLQUEST software (Becton Dickinson). Parameters were as follows: the amplifiers were set in linear mode for forward scattering, and in logarithmic mode for the green fluorescence detector (FL1, 530/30 nm band-pass filter) and the orange fluorescence detector (FL2, 585/21 nm band-pass filter). The amplifier gain was set at 1.00 for forward scattering; the detector voltage was set to E00 for forward scattering and 600 V for FL1, and the forward-scattering threshold was set at 52. The EGFP fluorescence signal was collected through a 530/30 nm band-pass filter (FL1), and the fluorescence intensity of 10 000 cells was defined as the FL1-height (FL1-H) geometric mean (see Fig. 3).

Growth assay to test mating ability

Each engineered yeast strain was cultivated in 5 mL of YPD medium with the mating partner BY4742 at 30 °C for 3 h, setting the initial D_{600} of each haploid cell at 0.1. After cultivation, yeast cells were harvested by centrifugation (3000 g, 5 min), and then washed with distilled water using centrifugation. To measure the range of mating ability of each strain, dilution series of yeast cell suspensions were prepared (D_{600} = 1.0, 0.1 and 0.01), and 10 µL of each suspension was spotted onto SD solid medium without methionine and lysine but containing 20 mg·L^{-1} histidine, 30 mg·L^{-1} leucine and 20 mg·L^{-1} uracil (SD-Met,Lys). Quantification of mating ability was performed by colony counting as follows. To obtain 100–1000 colonies on a plate, 1 mL of cell suspension was applied to SD-Met,Lys plates by selecting an appropriate dilution factor for each strain. The measured colony number was multiplied by each dilution factor to estimate the number of diploid cells generated by 1 mL of cell suspension, setting D_{600} at 1.0.

Screening of target cells from model libraries

Model libraries were prepared by mixing the target cells (FC3-2 or BZFG2118) with control cells (FC2-2 or BFG2Z18-WT) in the initial ratios shown in Table 3. These libraries were cultivated in 10 mL of YPD medium with mating partner BY4742 at 30 °C for 3 h, setting the initial D_{600} of each haploid cell at 0.1. After cultivation, yeast cells were harvested by centrifugation (3000 g, 5 min), and then washed with distilled water using centrifugation, applied to SD-Met,Lys plates and incubated at 30 °C for 2 days. Ten colonies were selected and separately grown in YPD medium overnight. The genomes were extracted from the cultivated yeast cells, and the coding region of the binding candidates was amplified by PCR using primers 5′-AAATATAAAACGCTAGCGTCGACATGGC GC-3′ and 5′-AGCGTAAAGGATGGGGAAAG-3′. The final ratio of target cells was determined by the number of colonies retaining the target gene divided by that of total colonies obtained on the diploid selectable medium, and the

screening efficiency was defined as the final ratio divided by the initial ratio of target cells (initial ratio of target cells is defined as the population of target cells in the prepared library).

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MINIREVIEW SERIES

Protein–protein interactions and selection

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Protein–protein interactions can be studied from the perspectives of biochemistry, signal transduction, and metabolic or genetic/epigenetic networks. Indeed, protein–protein interactions are at the core of the entire interactome of all living cells. Interactions between proteins are important for numerous biological functions. For example, signals from the exterior of a cell are mediated to the inside of that cell by protein–protein interactions between signaling molecules. Signal transduction plays a fundamental role in many biological processes and in many diseases, such as cancers. Information about these interactions improves our understanding of diseases and can provide a basis for new therapeutic approaches. In this series, three minireviews highlight methods under development for detecting and screening for protein–protein interactions. The overall goal of the series is to describe the methodology for elucidating protein–protein interactions, to learn more about protein interaction mechanisms, and to understand how to generate specific molecule-binding novel proteins.

The first minireview, by Ishii *et al.*, reports on yeast guanine nucleotide-binding protein signaling-based protein–protein interaction screening methods. These methods have been used to detect protein–protein interactions inside yeast cells, using cell growth or enzymatic reactions as readouts. These systems allow us to understand interactions inside cells under physiological conditions. Interestingly, quantification of the affinity between specific proteins is also possible. Recent progress in yeast-based systems for detecting and screening of protein–protein interactions is summarized.

In the second minireview, Mihara's group focuses on protein-detecting microarrays, and recent progress and useful applications are summarized. Protein array technology allows for the study of both the comprehensive proteome and focused proteomes in high-throughput screening. Protein–protein or protein–ligand interactions are also detectable using array technology. Miniaturized and parallelized array technology for protein detection is now being recognized as a powerful tool for predictive/early diagnosis.

Umetsu *et al.*, in the third minireview, describe constructive approaches for generating specific molecule-binding proteins. Antibodies are well known as having specific molecular recognition properties. In this minireview, the authors demonstrate the functionalization of small, nonantibody proteins on the basis of tertiary structures visualized by X-ray and NMR analyses. Approaches that combine peptide grafting and local libraries for designing high-affinity scaffold proteins are summarized.

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Protein–protein interactions and selection: yeast-based approaches that exploit guanine nucleotide-binding protein signaling

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For elucidating protein–protein interactions, many methodologies have been developed during the past two decades. For investigation of interactions inside cells under physiological conditions, yeast is an attractive organism with which to quickly screen for hopeful candidates using versatile genetic technologies, and various types of approaches are now available. Among them, a variety of unique systems using the guanine nucleotide-binding protein (G-protein) signaling pathway in yeast have been established to investigate the interactions of proteins for biological study and pharmaceutical research. G-proteins involved in various cellular processes are mainly divided into two groups: small monomeric G-proteins, and heterotrimeric G-proteins. In this minireview, we summarize the basic principles and applications of yeast-based screening systems, using these two types of G-protein, which are typically used for elucidating biological protein interactions but are differentiated from traditional yeast two-hybrid systems.

Introduction

Protein–protein interactions have fundamental roles in a variety of biological functions, and are of central importance for virtually every process in a living cell. Hence, many methodologies for elucidating protein interactions have been developed during the past couple of decades. To investigate interactions inside cells under physiological conditions, especially, yeast would be a most typical organism, and various *in vivo* selection approaches are now available.

The budding yeast *Saccharomyces cerevisiae* is one of the simplest unicellular eukaryotes, and is often used as a eukaryotic model organism for cellular and molecular biology [1–5]. Yeast has several benefits, including the possession of eukaryotic secretory machinery, post-translational modifications, rapid cell growth, and well-established and versatile genetic techniques. Thus, it is also used to establish technologies with which to survey interactions of eukaryotic

proteins. The yeast two-hybrid (Y2H) system, which was originally designed to detect protein–protein interactions *in vivo* by separation of a transcription factor into a DNA-binding domain and a transcription activation domain, is a typical representative of a yeast-based genetic approach [6], and numerous improved Y2H systems have been developed to overcome its potential problems [7–14]. The utility of Y2H systems has been demonstrated to varying degrees, involving analyses of comprehensive interactome networks [15–18], identification of novel interaction factors [19–22], investigations of homodimerization or hetero-dimerization [23–25], and the obtaining of conformational information [26–28]. Thus, yeast is definitely an attractive organism for analyzing the interactions of eukaryotic proteins.

Guanine nucleotide-binding proteins (G-proteins) are signaling molecules that are highly conserved among various eukaryotes, and that engage in a wide variety of cellular processes [3,29]. They switch from an inactive to an active state by exchanging a GDP molecule for GTP, and they return to the inactive state by hydrolyzing GTP to GDP. They are divided into two main groups: small monomeric G-proteins and heterotrimeric G-proteins [29]. Because eukaryotic yeast cells have both types of G-protein, but are not as complicated as higher eukaryotes, yeast has been used as the model organism for the study of G-protein machinery [30–32]. Much knowledge of G-protein signaling in yeast has been accumulated and used to study cellular processes, including protein interactions.

In this minireview series highlighting the methodologies for elucidating protein–protein interactions, the other two minireviews by K. Tomizaki *et al.* [33] and M. Umetsu *et al.* [34] deal with array based-technologies for detecting protein interactions *in vitro*, and constructive approaches to the generation of novel binding proteins on the basis of tertiary structural information, respectively. In this first minireview, we focus on and summarize the unique technologies used to exploit yeast G-protein signaling, which are commonly used for the exploration of biological protein interactions under physiological *in vivo* conditions but are distinguishable from conventional Y2H systems from a scientific and engineering perspective.

Ras signaling-based screening systems for protein–protein interactions

Small monomeric G-protein signaling in yeast

Small monomeric G-proteins, such as Ras and Ras-like proteins, are found mainly at the inner surface of the plasma membrane as monomers. They function as GTP-ases on their own, and are involved in controlling cell proliferation, differentiation, and apoptosis [29]. The Ras proteins are, in addition, necessary for the completion of mitosis and the regulation of filamentous growth [35]. In the yeast *S. cerevisiae*, growth and metabolism in response to nutrients, particularly glucose, is regulated to a large degree by the Ras–cAMP pathway [30,31,35]. Ras proteins activate adenylate cyclase, which synthesizes cAMP, and the increase in cytosolic cAMP levels activates the cAMP-dependent protein kinase, which has an essential role in the progression from the G_1 phase to the S phase of the cell cycle.

Owing to their intrinsically slow GTPase and GTP–GDP exchange activities, Ras proteins are strictly controlled by two classes of regulatory proteins: GTPase-activating proteins (GAPs), and guanine nucleotide exchange factors (GEFs) [35]. RasGAPs, which act as negative regulators of Ras–cAMP signaling by accelerating hydrolysis of GTP to GDP on Ras proteins, can stimulate the GTPase activity of Ras proteins to terminate the signaling event. On the other hand, RasGEFs, which contain Cdc25p and Sdc25p in yeast, stimulate the exchange of GDP for GTP on Ras proteins. The stimulated RasGEFs activate the Ras–cAMP signaling pathway. Whereas Cdc25p is essential in most genetic backgrounds, Sdc25p is dispensable and is normally expressed only during nutrient depletion or in nonfermentative situations. Through its role in regulating cAMP levels, Cdc25p is involved in fermentative growth, nonfermentative growth, cell cycling, sporulation, and cell size regulation. Thus, the main positive regulator of yeast Ras proteins is Cdc25p.

Characteristic aspects of Ras signaling-based screening systems

Ras signaling-based yeast screening systems for the exploration of protein interaction partners allow for positive selection of interactions between soluble cytosolic proteins or between a soluble protein and a hydrophobic membrane protein through the restoration of Ras signaling [36–38]. These systems employ the *cdc25* yeast strain, which is deficient in Ras signaling and regains it with the presence of interacting protein pairs. The machinery of intrinsic cell survival and proliferation of Ras signaling is utilized for the readout. Interactions of proteins of interest, including transcriptional activators or repressors that might induce transcription of a reporter or disable vital functions in yeast, can be investigated because of the restitution of Ras signaling on the plasma membrane but the absence of reconstitution of DNA-binding transcription factors

in the nucleus. The restricted cell survival with Ras signaling-based selection is suitable for screening large libraries (Table 1), although the method has comparative difficulty in accurately assessing relative interaction strengths.

Sos recruitment system

The Sos recruitment system (SRS) was initially reported as a Ras signaling-based screening system,

and it takes advantage of the fact that the human RasGEF protein, hSos, can substitute for the GEF of yeast endogenous Ras (yRas) protein, Cdc25p, to allow cell survival and proliferation (Fig. 1A) [36]. In the SRS, a yeast variant strain that has the temperature-sensitive *cdc25-2* allele is required. The *cdc25-2* strain cannot survive at a restrictive temperature (36 °C), owing to a lack of function of Cdc25p to activate Ras signaling, whereas it can grow at a lower temperature (25 °C). One protein should be

Table 1. Protein–protein interaction pairs identified or applied in G-protein signaling-based systems.

Interaction pair	Reference
Sos recruitment system	
c-Jun–JDP1 or c-Jun–JDP2 (Jun dimerization proteins)	[36]
c-Jun–Fra-2, c-Jun–FosB or c-Jun–c-Fos (Fos)	[36]
p110–p85	[36]
BRCA1 (breast cancer susceptibility gene 1)–CtIP (CtBP-interacting protein)	[84]
Sox9–PKA-Cα (protein kinase A catalytic subunit α)	[85]
VDAC1 (voltage-dependent anion-selective channel 1)–Tctex1 (t-complex testis expressed-1)	[86]
VDAC1–PBP74 (peptide-binding protein 74)	[86]
p5–p5	[87]
GABA$_A$ receptor γ2 subunit–GODZ (Golgi-specific DHHC zinc finger protein)	[88]
IRS-1 (insulin receptor substrate 1)–HDAC2 (histone deacetylase 2)	[89]
p73–PKA-Cβ (protein kinase A catalytic subunit β)	[90]
Truncated ERβ (estrogen receptor β)–truncated ERβ	[91]
HBO1 (histone acetyltransferase binding to ORC-1)–PR (progesterone receptor)	[92]
CMV 1a (cucumber mosaic virus 1a)–TIP1 or CMV 1a–TIP2 (tonoplast intrinsic proteins)	[93]
TRAF2 (tumor necrosis factor receptor associated factor 2)–Smurf2 (SMAD-specific E3 ubiquitin protein ligase 2)	[94]
EF3 (elongation factor 3)–Cch1 (high-affinity calcium channel)	[95]
Ras recruitment system	
c-Jun–c-Fos	[38]
p110–p85	[38]
JDP2–C/EBPγ (CCAAT/enhancer-binding protein)	[38]
Pac65 (Pac2; p21-activated kinase 2)–Rac1 mutant	[38]
Pac65–Grb2 (growth factor receptor-binding protein 2)	[38]
Sos (son of sevenless)–Grb2 (growth factor receptor-bound protein 2)	[38]
Truncated EGFR (epidermal growth factor receptor) fused with M-Jun–truncated EGFR fused with M-Fos[a]	[39]
Glucocorticoid receptor NR3C1–ZKSCAN4 (zinc finger with KRAB and SCAN domains 4)	[40]
PacR (Pac2 regulatory domain)–Chp (Cdc42Hs homologous protein)	[96]
β-Catenin–CBP (CREB-binding protein)	[97]
JNK (c-Jun N-terminal kinase)–IKAP (IκB kinase complex-associated protein)	[98]
ErbB (EGFR)–Grb2	[99]
c-Myc–Krim-1A or c-Myc–Krim-1B (Krab box proteins interacting with Myc)	[100]
RalA (Ras-like protein A)–ZONAB (ZO-1-associated nucleic acid-binding protein)	[101]
Yeast–mammal chimeric Gα system	
Snf1 (AMP-activated protein kinase)–Snf4 (regulatory subunit of Snf1 kinase complex)	[78]
Raf–Ras mutant	[78]
Gγ interfering system (G-protein fusion system)	
Syntaxin 1a–nSec1 (neuronal Sec1)	[79]
FGFR3 (fibroblast-derived growth factor receptor 3)–SNT-1 (FGFR signaling adaptor)	[79]
Gγ recruitment system	
ZZ domain or Z variants (Z domain: B domain mutant derived from protein A)–Fc part (of human IgG)	[80]
Competitor-introduced Gγ recruitment system[b]	
ZZ domain or Z variants–Fc part	[102]

[a] This system is to be used for monitoring receptor tyrosine kinase activity. [b] This system is to be used for selective isolation of affinity-enhanced variants.
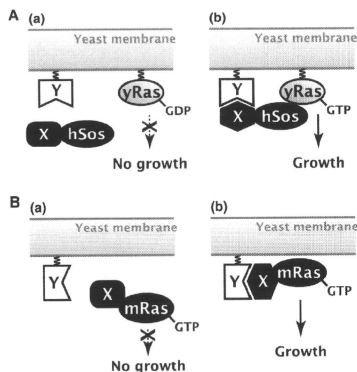
Fig. 1. Schematic illustration of Ras signaling-based screening systems. (A) The SRS system using the human RasGEF protein, hSos. (a) Noninteracting protein pairs are unable to activate the yeast Ras signaling pathway, and are also unable to drive cell growth. (b) Interacting protein pairs bring hSos to the plasma membrane, where it can exchange GDP for GTP of yeast endogenous Ras. The active form of GTP-bound yRas allows cell survival. (B) The RRS system using a constitutively active mutant of mammalian Ras lacking the lipid modification motif (mRas). (a) Noninteracting protein pairs are unable to activate the yeast Ras signaling pathway, and are also unable to drive cell growth. (b) Interacting protein pairs bring mRas to plasma membrane, where it can activate the yeast Ras signaling pathway. Ras signaling allows cell survival. X and Y represent test proteins for interaction analysis.

membrane-associated or be attached to an inner membrane translocating signal involved in myristoylation and palmitoylation, and the other protein should be soluble and be fused to hSos to prevent false autoactivation by membrane localization of hSos. Only when the membrane-localized protein interacts with the hSos fusion protein will hSos be recruited to the plasma membrane and yeast Ras signaling be rescued. As a consequence, the temperature-sensitive mutant that expresses interacting protein pairs can grow at 36 °C.

Using the SRS, a novel repressor that interacts with the c-Jun subunits of AP-1 and represses its activity was isolated [36] (Table 1). AP-1 is a transcription factor that binds to DNA through a leucine zipper motif. Thus, the ability of the SRS to identify transcriptional regulators has been reasonably well established, owing to the membrane-localized interaction, unlike conventional Y2H systems based on the reconstitution of DNA-binding transcription factors in the nucleus.

Ras recruitment system

The Ras recruitment system (RRS), using mammalian Ras (mRas), was later developed as an improved version of the SRS [38]. The RRS has the advantages of the SRS without some of its limitations. For example, the RRS permits more strict selection, owing to the stringent requirement for membrane localization of mRas, can eliminate the isolation of predictable Ras false positives, owing to the introduction of mRasGAP, and can more broadly detect interactions, owing to the relatively small size of Ras as compared with hSos [37,38]. The RRS is based on the absolute requirement that Ras be localized to the plasma membrane for its function (Fig. 1B). In the RRS, mRas lacking its CAAX motif for localization to the plasma membrane, but possessing a constitutively active mutation, is used as a substitute for hSos, and mRasGAP is additionally expressed. The membrane localization of mRas through protein–protein interactions in a *cdc25-2* yeast strain results in the activation of its downstream effector, adenylyl cyclase, and restores its growth ability. In an initial report, the usefulness of the RRS was confirmed by practical screening of a cDNA library of 500 000 independent transformants [38] (Table 1). Later, the RRS was applied to detect the activity and inhibition of a dimerization-dependent receptor tyrosine kinase and to identify an interacting pair of human glucocorticoid receptors from a HeLa cell cDNA library [39,40] (Table 1).

Pheromone signaling-based screening systems

Heterotrimeric G-protein signaling in yeast

As peripheral membrane proteins, heterotrimeric G-proteins associate with the inner side of the plasma membrane. Heterotrimeric G-proteins consisting of three subunits, Gα, Gβ, and Gγ, exist in various subfamilies and are widely conserved among eukaryotic species. They transduce messages from ubiquitous receptors, which control important functions such as taste, smell, vision, heart rate, blood pressure, neurotransmission, and cell growth [29]. Yeast has only two types of heterotrimeric G-protein: pheromone signaling-related and nutrient signaling-related [30–32]. Nutrient signaling is profoundly and intricately linked to Ras signaling [30,31], whereas the pheromone signaling pathway is connected to mating processes [32].

The yeast pheromone signaling-related G-protein comprises three subunits, Gpa1p, Ste4p, and Ste18p, which structurally correspond to mammalian Gα, Gβ,

and Gγ, respectively [32]. The heterotrimeric G-protein is divided into two key components from the perspective of structure and function. Gα (Gpa1p) is associated with the intracellular plasma membrane through dual lipid modifications of myristoylation and palmitoylation in the N-terminus [41], whereas the Gβγ dimer (the Ste4p–Ste18p complex) is also localized to the inner leaflet of the plasma membrane through dual lipid modifications of farnesylation and myristoylation in the C-terminus of Ste18p, and the formation of a

complex between Ste4p and lipidated Ste18p [41,42]. They form part of the signaling cascade activated by G-protein-coupled receptors (GPCRs), and mediate cellular processes in mating in response to the presence of pheromone (Fig. 2A).

The yeast haploid a-cell has a sole pheromone receptor, Ste2p, which is classified as a GPCR, and the tridecapeptide α-factor functions as a pheromone and binds to the Ste2p receptor on the cell surface [32]. The heterotrimeric G-proteins are closely associated
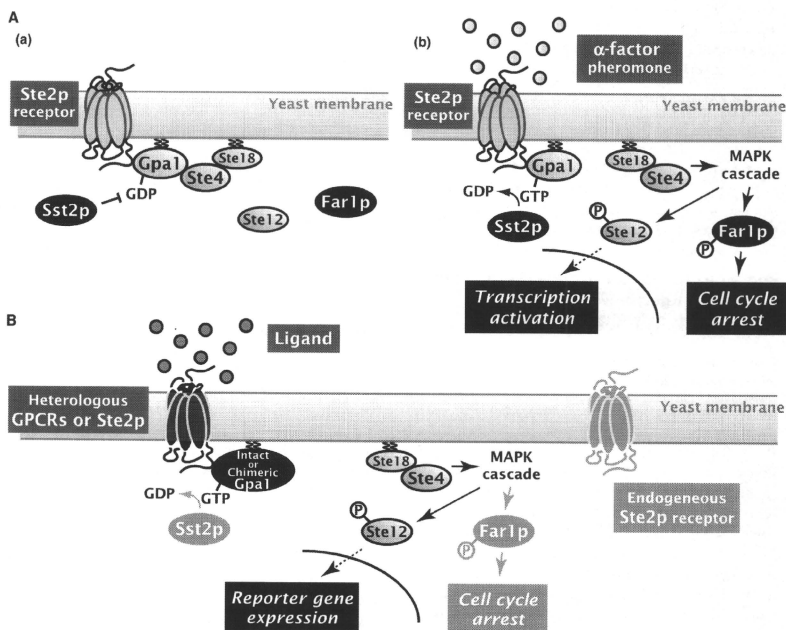

Fig. 2. Yeast pheromone signaling pathway and its utilization for a GPCR biosensor. (A) Schematic illustration of the pheromone signaling pathway. (a) In the absence of α-factor, heterotrimeric G-protein is unable to activate the pheromone signaling pathway. (b) Binding of α-factor to Ste2p receptor activates the pheromone signaling pathway through heterotrimeric G-protein. Sequestered Ste4p–Ste18p complex from Gpa1p activates effectors and subsequent kinases that constitute the MAPK cascade, resulting in phosphorylation of Far1p and Ste12p. Phosphorylation of Far1p leads to cell cycle arrest. Phosphorylation of Ste12p induces global changes in transcription. Sst2p stimulates hydrolysis of GTP to GDP on Gpa1p, and helps to inactivate pheromone signaling. (B) Schematic illustration of typical genetic modifications enabling the pheromone signaling pathway to be used as a biosensor to represent activation of GPCRs. Intact or chimeric Gpa1p can transduce the signal from yeast endogenous Ste2p or heterologous GPCRs that are expressed on the yeast plasma membrane. Transcription machineries that are closely regulated by the phosphorylated transcription factor, Ste12p, are used to detect activation of pheromone signaling with various reporter genes. FAR1, SST2 and STE2 are often disrupted (shown in light gray) to prevent growth arrest, improve ligand sensitivity, and avoid competitive expression of yeast endogenous receptor.

with the intracellular domain of the Ste2p receptor, and the pheromone-bound receptor is conformationally changed and activates the G-protein [43]. Gpa1p is thereby changed from an inactive GDP-bound state to an active GTP-bound state and dissociates the Ste4p–Ste18p complex. Subsequently, the dissociated Ste4p–Ste18p complex binds to effectors through Ste4p, and then activates the mitogen-activated protein kinase (MAPK) cascade [44,45]. The Ste5 scaffold protein binds to the kinases of the MAPK cascade and brings them to the plasma membrane. The concentration of the bound kinases on the membrane possibly promotes amplification of the signal [46,47]. As a consequence, the activated pheromone signaling leads to the phosphorylation of Far1p and the transcription factor Ste12p. These phosphorylated proteins trigger cell cycle arrest in G_1 [48–50] and global changes in transcription [51,52]. *FUS1* gene expression is representative of the drastic changes in transcription in response to pheromone signaling [53,54]. As a principal negative regulator, the Gpa1-specific GAP Sst2p, a member of the regulator of G-protein signaling family, is also involved in the pathway [55,56].

Pheromone signaling-based screening systems – ligand–GPCR or GPCR–G-protein interactions

Background of pheromone signaling-based screening systems

GPCRs constitute the largest family of integral membrane proteins, and have a variety of biological functions. They are the most frequently addressed drug targets, and modulators of GPCRs form a key area for the pharmaceutical industry, representing nearly 30% of all Food and Drug Administration-approved drugs [57,58]. Yeast permits the functional expression of various heterologous GPCRs and other signaling molecules such as G-proteins. Yeast also facilitates versatile genetic techniques for screening and quantification. Therefore, it offers opportunities to establish fundamental technologies for drug discovery or basic medicinal study [59,60]. Yeast-based screening systems exploiting pheromone GPCR signaling enable the analysis of several interactions, including not only protein–protein but also ligand–receptor and receptor–protein interactions. These systems can recognize the on–off switching of a signal, such as the binding of a agonist/antagonist to a receptor, and critical mutations involved in ligand-dependent or constitutive activation/inactivation of signaling molecules. In addition, assays can be performed at the yeast optimum

temperature of 30 °C, unlike with Ras signaling-based systems, which require the incubation of yeast cells at suboptimal temperatures (25 and 36 °C), and the monitoring or discrimination of the signaling changes through quantitative and survival readouts. Hence, they have been applied in various experiments, including target identification, ligand screening, and receptor mutagenesis.

Pheromone signaling as a biosensor for understanding GPCRs

GPCRs have a common tertiary structure, composed of seven hydrophobic integral membrane domains, and the mechanism of signaling that is mediated by heterotrimeric G-proteins is also conserved between yeast and mammalian cells. This has led to the construction of ingenious systems that provide for the mutual exchange of signals between heterologous GPCRs and yeast G-proteins in yeast without generating dysfunctions. With versatile screening techniques, yeast can be used as a sensor to detect the initiation of GPCR-associated signaling [59,60]. Briefly, in wild-type yeast **a**-cells, Ste2p receptor or mammalian receptors can activate the yeast pheromone signaling pathway via intracellular heterotrimeric G-proteins, including the native form or an engineered form of Gpa1p, in response to ligand binding. The activated pheromone signals cause cell cycle arrest and transcription activation, which are exploited as signaling readouts (Fig. 2A,B). These biosensing techniques have been established in yeast with engineered pheromone signaling, and numerous characteristics of pheromone signaling molecules have been successfully elucidated [43–45, 47–50, 53–55]. Moreover, pheromone signaling-related molecules, such as Ste2p receptor, G-proteins, and peptidic α-factor pheromone, have been extensively mapped with mutagenesis techniques, demonstrating their usefulness for screening huge libraries and for identification of important domains or amino acids [61–66].

Bioassay and transcriptional assay for signaling detection

The arrest of the cell cycle completely prevents cell growth during signaling. Monitoring of cell densities in liquid media with or without pheromones can distinguish signaling on the basis of delay of entry into the logarithmic growth phase. The agar diffusion bioassay (halo assay), in which cells are mixed with unsolidified fresh agar medium in which pheromone-spotted paper filter disks are placed, can also discriminate signaling by showing cleared-out areas around the disks,

forming halos, owing to the robust inhibition of cell growth (the halos may look blacked out on a monochromatic figure) [55,62,63,66,67].

On the other hand, the use of transcriptional changes that are closely regulated by the signaling makes possible versatile procedures for detection. The *FUS1* gene, which is engaged in drastic augmentation of the transcription level responding to the signal, is commonly taken as a reflector of signaling and is fused with various reporter genes associated with growth and photometry. Auxotrophic or drug-resistant reporter genes, such as *HIS3* or *hph*, are generally used for selection, and are suitable for screening large-scale libraries [66–68]. Colorimetric, luminescent and fluorescent reporters, such as *lacZ*, *luc*, or *GFP*, are usually used for numerical conversion and are appropriate for relative and quantitative assessment of signaling levels [61–64,66–68].

Gene disruption for system modification

The arrest of the cell cycle caused through phosphorylation of Far1p allows for the examination of pheromone signaling [55,59,60,62,63,66,67]. However, this makes growth reporter genes for positive selection, such as *HIS3*, useless for the detection of signaling, owing to stagnation of cell growth [66,67], whereas the synchronization of the cell cycle in G_1 arrest provides uniform levels of expression of reporter genes such as *GFP* for each cell [69]. For that reason, *FAR1* is usually disrupted in positive selection screens using growth selection (Fig. 2B). Because the *far1Δ* strain never induces cell cycle arrest, it can be used in growth selection to screen for positive clones in response to pheromone signaling, which is represented by the expression of the *HIS3* reporter gene on histidine-defective plates [66,67]. At the same time, it has been reported that the arrest of the cell cycle causes the drastic dropout of episomal plasmids, resulting in a serious problem when the library is screened and the target plasmids are collected, and hence the disruption of *FAR1* could significantly improve plasmid retention rates [69]. Accordingly, disruption of *FAR1* is required for positive growth screening.

The *SST2*-deficient strategy is widely used in utilizing pheromone signaling as a sensor, owing to hypersensitivity for ligand binding [59,60,63,67,69]. *SST2* gene encodes the Gpa1p-specific GAP that stimulates hydrolysis of GTP to GDP on Gpa1p and helps in the inactivation of pheromone signaling. Removal of Sst2p function causes a considerable decrease in GTPase activity for Gpa1p, and makes the conversion of GTP to GDP difficult, owing to a lack of competence of

GTPase activity (Fig. 2B). The loss of *SST2* could provide supersensitivity, even to a 250–10 000-fold lower concentration of α-factor [67]. However, a relatively high background signal of the *sst2Δ* strain, especially when grown in rich medium such as YPD, has been confirmed in the absence of α-factor pheromone by a transcription assay using the *FUS1–GFP* reporter gene [69]. Although the *SST2*-deficient strategy is a powerful technology for experiments requiring high sensitivity, it does not necessarily produce the best signal-to-noise ratio. Accordingly, choosing the correct situation for using Sst2p is required for each experiment. In addition, *STE2* is often disrupted, to avoid competitive expression of yeast endogenous receptor [59–64,66,69].

Expression of heterologous GPCRs

Many heterologous GPCRs containing adrenergic, muscarinic, serotonin, neurotensin, somatostatin, olfactory and many other receptors have been successfully expressed in yeast, and the feasibility of yeast-based GPCR screening systems has been demonstrated [59,60,68,70–75]. Yeast Gpa1p, which is equivalent to Gα, shares high homology, in part, with human $Gα_i$ classes, and a number of GPCRs of human and other species are able to interact with Gpa1p and activate pheromone signaling in yeast [73–75]. Many other human GPCRs can also function as yeast signaling modulators as a result of various genetic modifications, including one in which chimeric Gpa1p systems (so-called 'transplants') have only five amino acids in the C-terminus of Gpa1p substituted for those of human Gα subunits, including the $Gα_{i/o}$, $Gα_s$ and $Gα_q$ families (Fig. 2B) [71]. Indeed, these transplants have allowed functional coupling of serotonin, muscarinic, purinergic and many other receptors to the yeast pheromone pathway [71–73,76].

The rat M_3 muscarinic acetylcholine receptor has been used for rapid identification of functionally critical amino acids, with random mutagenesis of the entire sequence [72]. In this system, the *CAN1* reporter gene coding for arginine–canavanine permease was integrated into the locus of a pheromone response gene in yeast cells whose endogenous *CAN1* gene was deleted, and the recombinant strain expressed Can1p in response to ligand-dependent signaling. Owing to the cytotoxicity of canavanine caused by Can1p expression, recombinant strains with inactivating mutations in the receptor can survive on agar media containing canavanine and receptor-specific agonists. The recovered mutant M_3 muscarinic acetylcholine receptors in this system also show substantial functional impairments in transfected mammalian cells, and the utility