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Membrane-displayed peptide ligand activates the pheromone response pathway in *Saccharomyces cerevisiae*

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The budding yeast, *Saccharomyces cerevisiae*, is an attractive host for studying G protein-coupled receptors (GPCRs). We developed a system in which a peptide ligand specific for GPCR is displayed on yeast plasma membrane. The model system described here is based on yeast plasma membrane display of an analogue of α-factor, which is a peptide ligand for Ste2p, the GPCR that activates the yeast pheromone response pathway. α-Factor analogues, containing linkers of varying lengths and produced in yeast cells, became attached to the cell plasma membrane by linking to the glycosylphosphatidylinositol (GPI)-anchored plasma membrane protein Yps1p. We were able to demonstrate that an optimized α-factor analogue activated the pheromone response pathway in *S. cerevisiae*, as assessed by a fluorescent reporter assay. Furthermore, it was shown that linker length strongly influenced signalling pathway activation. To our knowledge, this is the first report documenting functional signalling by a plasma membrane-displayed ligand in *S. cerevisiae*.

Keywords: GPI-anchored plasma membrane protein/ligand-GPCR interaction/membrane-displayed ligand/yeast GPCR assay/yeast pheromone response pathway.

Abbreviations: EGFP, enhanced green fluorescent protein; GPCR, G protein-coupled receptors; GPI, glycosylphosphatidylinositol; GPI-CWP, GPI-anchored cell wall protein; GPI-PMP, GPI-anchored plasma membrane protein; MAPK, mitogen-activated protein kinase; TM, transmembrane domain.

G protein-coupled receptors (GPCRs) comprise seven transmembrane domain proteins that are encoded by one of the largest gene families in the human genome (*1*). These proteins participate in virtually all aspects of cellular physiology, including hormonal responses, neuronal transmission and mediation of taste, smell and vision (*2*).

The GPCR assay using the baker's yeast *Saccharomyces cerevisiae* has been established as an experimental system for characterizing human receptor pharmacology and signal transduction mechanisms (*3*, *4*). Its attractive features are simplicity in genetic manipulations, economical propagation of yeasts, easy maintenance of stably expressing cell lines, availability of an evolutionarily highly conserved signalling pathway and only two endogenous GPCRs that can be readily eliminated, and numerous documented successes in producing functional mammalian GPCRs (*5*, *6*).

We have demonstrated heterologous production of the metabotropic glutamate receptor subtype 1 on the *S. cerevisiae* plasma membrane (*7*). We also have developed a yeast cell-surface engineering system (*8*, *9*), which enables display of proteins or peptides on yeast cell walls linked to a glycosylphosphatidylinositol (GPI)-anchored cell wall protein (GPI-CWP). To study signalling through transmembrane receptors, however, it was obviously necessary to develop a peptide-ligand display system based on the plasma membrane.

Yps1p is a representative GPI-anchored plasma membrane protein (GPI-PMP) (*10*). It is suggested that the sequence of the five amino acid residues within the upstream region of the GPI anchor addition site (the ω site) is the primary determinant of whether a GPI protein localizes to the cell wall or to the plasma membrane (*11*). By utilizing the anchoring domain of Yps1p, we reasoned that proteins or peptides displayed will remain on the outer surface of the plasma membrane, compared to cell wall localization of cell-surface display systems utilizing the flocculation domain of Flo1p or the C-terminal domain of α-agglutinin (*12*).

Here, we established a yeast membrane-displayed peptide ligand system using Yps1p as an anchoring protein. The α-factor, a 13-amino acid residue-long peptide agonist of the yeast pheromone response pathway, was displayed on plasma membranes with varying linker lengths. We were successful in demonstrating that the membrane-displayed α-factor functionally activated the pheromone response pathway, and that the linker length was crucially important for signalling. This is the first report demonstrating a membrane-displayed peptide ligand using *S. cerevisiae*. This will be a new platform for identifying novel peptide ligands for both liganded and orphan mammalian GPCRs.

Materials and Methods

Strains and media

The haploid yeast (*S. cerevisiae*) strain W303-1A (*MAT*a; *ade2-1*; *his3-11,15*; *leu2- 3,112*; *trp1-1*; *ura3-1*) (*13*) and other strains constructed specifically for the present study and their genotypes are listed in Table I. Yeast strains were cultivated aerobically in yeast peptone dextrose (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose] or synthetic dextrose (SD) medium [0.67% (w/v) yeast nitrogen base without amino acids, 2% glucose and appropriate supplements]. Agar (2% w/v) was added to these media to produce YPD and SD solid media. *Escherichia coli* DH5α (TOYOBO, Osaka, Japan) was used as a host for recombinant DNA manipulation and grown in Luria–Bertani medium [1% (w/v) tryptone, 0.5% yeast extract (w/v) and 0.5% sodium chloride (w/v)] containing 100 μg ml^{-1} ampicillin. After selection, a single colony was used to isolate the plasmid for yeast transformation.

Expression vectors

The polymerase chain reaction (PCR) was performed using KOD-plus-DNA polymerase (Toyobo, Osaka, Japan). The reporter plasmid pYEX-fet was constructed as follows: The *FUS1* promoter fragment was amplified from W303-1A genomic DNA using primers containing 5′-HindIII (5′-CCTaagcttTTGTATCTCTGCAGGATG CC-3′) and 3′-SalI (5′-TGCgtcgacGATTTTCAGAAACTTGAT GG-3′) recognition sites. The resulting fragment was inserted between the HindIII and SalI sites in pYEX-BX (Clontech, Mountain View, CA, USA), resulting in the plasmid pYEX-f. The *HIS3* gene in pFUS1-EGFP-HIS3 [generously provided by J. Ishii *et al.* (*14*)] was deleted by self-ligation after BamHI digestion. Using the resultant plasmid as a template, the *EGFP-FUS1* fragment with the terminator sequence was amplified with primers containing 5′-SalI (5′-ATAgtcgacATGGTGAGCAAGGGCGAGGA-3′) and 3′-EcoRI (5′-TATgaattcATGGTTACATAAAAGCTGCTTTGCC -3′) recognition sites and inserted into the SalI and EcoRI sites in pYEX-f. The resulting plasmid was designated pYEX-fet. pYEX-fet contained the selectable markers *URA3* and *leu2-d*. The multicopy plasmid containing *leu2-d* can be stably and highly amplified (*15*).

Plasmids for producing membrane-displayed α-factor with different linker lengths were constructed as follows: DNA fragments encoding FLAG Tag and the *YPS1* anchoring domain were amplified from W303-1A genomic DNA with primers containing 5′-BamHI (5′- GCGggatccGATTACAAGGATGACGATGACAA GGGTGGATCTGGTGGCACATCAAGTAAAAGAAATGTTG GTGATC) and 3′-SalI (5′- TGGCCGAgtcgacTCAGATGAATGC AAAAAGAAGAGAAATTAATG-3′) recognition sites, and inserted into the BamHI and SalI sites of pWGP3 (*16*), resulting in plasmid pYS0. Secondly, DNA fragments with additional different lengths of linker were amplified from pYS0 with primers including 5′-BamHI (5′-GCGggatcc<u>GGTGGATCT</u>GATTACAAGGATGAC GATGACAAG), where the underlined linker sequence varies depending on linker length [*e.g.* <u>GGT</u> for linker length 1, <u>GGTGGA</u> for linker length 2, <u>GGTGGAATC</u> for linker length 3 and (<u>GGTGG AATC</u>)$_2$ for linker length 6], and 3′-SalI (5′- TGGCCGAgtcgacTCA GATGAATGCAAAAAGAAGAGAAATTAATG-3′) sites and inserted into the BamHI and SalI sites of pYS0, resulting in plasmids pYS1, pYS2, pYS3, pYS4, pYS5, pYS6, pYS7, pYS8, pYS9, pYS10, pYS11, pYS12, pYS13, pYS15 and pYS21. Finally, a DNA fragment encoding the glucoamylase secretion signal sequence and the α-factor sequence was amplified from pULD1 (*15*) with primers possessing 5′-KpnI (5′-CGGggtaccATGCAACTGTTCAATTTGCCA TTG-3′) and 3′-BamHI (5′-CGCggatccACCGTACATTGGTTGG CCAGGTTTTAGTTGCAACCAATGCCAGGCAGAAACGAG CAAAGAAAAG-3′) recognition sites and inserted into the KpnI and BamHI sites in pYS0, pYS1, pYS2, pYS3, pYS4, pYS5, pYS6, pYS9, pYS12, pYS15 and pYS21. The resulting plasmids were designated as pYS0-αF, pYS1-αF, pYS2-αF, pYS3-αF, pYS4-αF, pYS5-αF, pYS6-αF, pYS9-αF, pYS12-αF, pYS15-αF and pYS21-αF. Additionally, the DNA fragment encoding the glucoamylase secretion signal sequence (lacking the α-factor sequence) was amplified similarly using the primer 3′-BamHI (5′- CGCggatccA CCGGCAGAAACGAGCAAAGAAAAGTAAG) and inserted into the KpnI and BamHI sites in pYS0. The resulting plasmid was named pYS0-nega and used as a negative control.

Construction of yeast strains

All yeast transformation procedures were conducted with Frozen-EZ Yeast Transformation-II kit (Zymo Research Corporation, Irvine, CA, USA). The WH-1 strain, carrying disrupted *SST2*, was obtained using the two-step procedure of Gueldener *et al.* (*17*) as follows: The *SST2* of W303-1A was disrupted using the *loxP-URA3-loxP* cassette amplified from pUG72 (*17*) with primers 5′- GAGGC GTTATAGGTTCAATTTGGTAATTAAAGATAGAGTTGTAA Gcagctgaagcttcgtacgc-3′ and 5′- TGTTTGTGCAATTGTACCTGA AGATGAGTAAGACTCTCAATGAAAgcataggccactagtggatctg-3′. Next, Ura-derivatives of the transformants were selected on 5-fluoroorotic acid media (*18*). Both the *SST2* disruption at the targeted locus and removal of *URA3* were verified by a diagnostic PCR technique with appropriate primers. This two-step procedure allows repeated use of *URA3*-selection for multiple gene disruptions. The WH-3 strain, carrying *SST2* and *FAR1* gene disruptions, was obtained in the same way with primers 5′- TAGATCCACTGGAAAG CTTCGTGGGCGTAAGAAGGCAATCTATTAcagctgaagcttcgtac gc-3′ and 5′- AGGAAAAGCAAAAGCCTCGAAATACGGGCCT CGATTCCCGAACTAgcataggccactagtggatctg-3′. The WH-5 strain, carrying *SST2*, *FAR1* and *BAR1* disruptions, was obtained in the same manner with primers 5′- CTAAAATCATACCAAAATAAAA AGAGTGTCTAGAAGGGTCATATAcagctgaagcttcgtacgc-3′ and 5′- ATATTTGATATTTATATGCTATAAAGAAATTGTACTCC AGATTTCgcataggccactagtggatctg-3′. The WH-5 strain was transformed with pYEX-fet, resulting in the WTH-5 strain. This WTH-5 strain was used as a host for the expression of the plasmids encoding the ligands. The WTH-5 strain was transformed with one of the ligand-encoding plasmids whose construction was described above.

Cultivation conditions

To prepare starter cultures, a series of WTH-5 strains with one of the membrane-displayed ligand plasmids were grown in 10 ml SD-Leu,Trp medium at 30°C overnight. The cultures used for the assays were initiated by inoculating the starter culture into 10 ml SD-Leu,Trp medium to give an initial optical density (OD) of 0.05 at 600 nm using a VMax Kinetic ELISA Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). These cultures were grown at 30°C on a rotatory shaker (250 rpm) for 21 h.

Plate assay

Two hundred microlitres of the main culture, 21 h after inoculation, was transferred to a 96-well plate (353072; BD Falcon), and the fluorescence was measured with a Fluoroskan Ascent Fluorometer (Labsystems OY, Helsinki, Finland). A filter pair with excitation at 485 nm and emission at 527 nm was used to detect the fluorescence of enhanced green fluorescent protein (EGFP) produced in the yeast cells. The OD^{600} of each well of the sample plate was measured simultaneously. In order to normalize the number of cells in each well, the fluorescence value was divided by the OD^{600}. The fluorescence of the

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

Strain	Genotype	Reference
W303-1A	MATa; *ade2-1; his3-11,15; leu2- 3,112; trp1-1; ura3-1*	(*13*)
WH-1	MATa; *ade2-1; his3-11,15; leu2- 3,112; trp1-1; ura3-1; sst2*Δ	Present study
WH-3	MATa; *ade2-1; his3-11,15; leu2- 3,112; trp1-1; ura3-1; sst2*Δ*: far1*Δ	Present study
WH-5	MATa; *ade2-1; his3-11,15; leu2- 3,112; trp1-1; ura3-1; sst2*Δ*: far1*Δ*: bar1*Δ	Present study
WTH-5	MATa; *ade2-1; his3-11,15; leu2- 3,112; trp1-1; ura3-1; sst2*Δ*: far1*Δ*: bar1*Δ: pYEX-fet	Present study

negative control well was subtracted from those of all the other wells (pYS-nega strain, see 'Expression vectors' section).

Fluorescence microscopy

The cells were observed with an inverted microscope (IX71; Olympus, Tokyo, Japan). The green EGFP fluorescence was detected using a U-MNIBA2 mirror unit with a BP470-490 excitation filter, DM505 dichroic mirror, and BA510-550 emission filter (Olympus).

FACS analysis

The yeast cells were suspended in PBS (pH 7.2) and analysed using a flow cytometer (JSAN; Bay Bioscience, Kobe, Japan). The fluorescence emission of 10,000 yeast cells at 535 ± 23 nm excited at 488 nm was plotted.

Isolation of yeast cell plasma membranes

Plasma membrane fraction was prepared as previously described method (*11*). Cells were grown overnight in SD-Leu,Trp medium. Cells were collected by centrifugation and resuspended in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA), and a protease inhibitor cocktail for use with fungal and yeast extracts (P8215: Sigma-Aldrich, St Louis, MO, USA) was added according to the manufacturer's instructions. A half volume of glass beads (diameter 0.5 mm) was added and placed in a Bead Smash (WAKENYAKU, Kyoto, Japan). The cells were vortexed at 4,000 rpm shaking for 1 min at 4°C three times, with 2 min immersion in ice in between each vortexing. The extract was centrifuged at 15,000*g* for 10 min to pellet all cell wall and membrane material. After removing the supernatant, the pellet was then boiled for 10 min in 50 mM Tris-HCl, pH 7.5, containing 2% (w/v) SDS. The SDS-soluble material was saved as a membrane fraction.

Western blot analysis

Membrane proteins were separated by electrophoresis on 15% (w/v) SDS polyacrylamide gels and electro-transferred onto a nitrocellulose membrane (Bio-Rad, Richmond, CA, USA). The membrane was blocked with 5% (w/v) skim milk powder in 2% (v/v) Tween/PBS overnight. Membranes were incubated for 1 h at room temperature with mouse anti-FLAG M2 antibody (Sigma-Aldrich) at a concentration of 1:10,000 in 5% skim milk/PBS. The blots were washed five times in 2% (v/v) Tween/PBS for 5 min, and antibody binding was detected using ECL-Plus (GE Healthcare, Buckinghamshire, UK).

Results

We first examined the anchoring domain to be used for ligand display. We used the C-terminal half of α-agglutinin (*8*, *9*), the long anchoring domain of Yps1p (51 amino acid residues including the ω site) (*11*), and the short anchoring domain of Yps1p (6 amino acid residues including the ω site). Of these, functional activation was observed only for the short anchoring domain of Yps1p. No fluorescence was observed in the system using the C-terminal-half of α-agglutinin or the long anchoring domain of Yps1p (data not shown).

An overview of our membrane display system is illustrated in Fig. 1. The membrane-displayed peptide consists of (i) a signal peptide for targeting to the secretory pathway, (ii) a peptide ligand for yeast endogenous GPCR Ste2p (α-factor), (iii) different lengths of flexible peptide linkers, (iv) a FLAG epitope tag and (v) a truncated region of the GPI-anchored plasma membrane protein Yps1p (Fig. 2). During protein processing within the secretory pathway, the signal peptide and GPI targeting sequences are cleaved, leaving the displayed ligand with a free N-terminus and the C-terminus covalently bound to GPI, whose acyl chains are inserted into the plasma membrane (Fig. 2B and C). Plasmids with several lengths of linkers were constructed because prediction

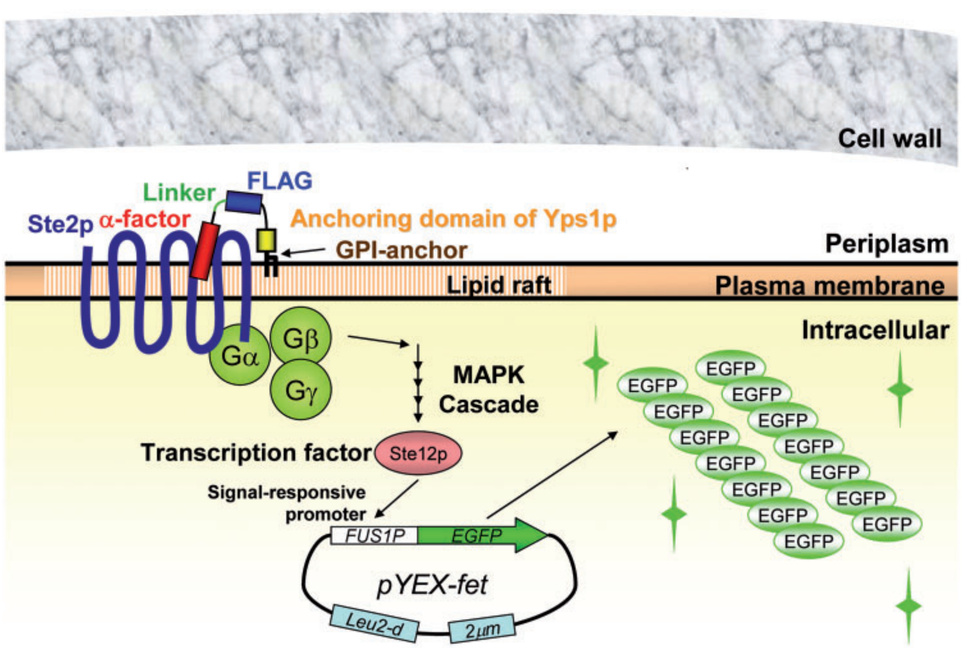

Fig. 1 Illustration of the peptide display system. Membrane-displayed α-factor (peptide agonist of the yeast pheromone response pathway) activates the pheromone receptor Ste2p, which leads to activation of G proteins, the MAPK cascade and the transcription factor Ste12p. Phosphorylated Ste12p induces overproduction of EGFP by binding to pheromone response element in the *FUS1* promoter of pYEX-fet (signal detection plasmid), which allows monitoring and quantification of signal transduction by fluorescence. To optimize signal output, gene disruption was used to delete *SST2* (to reduce desensitization and enhance signalling), *FAR1* (to prevent cell cycle arrest as a consequence of signalling) and *BAR1* (to prevent degradation of α-factor).

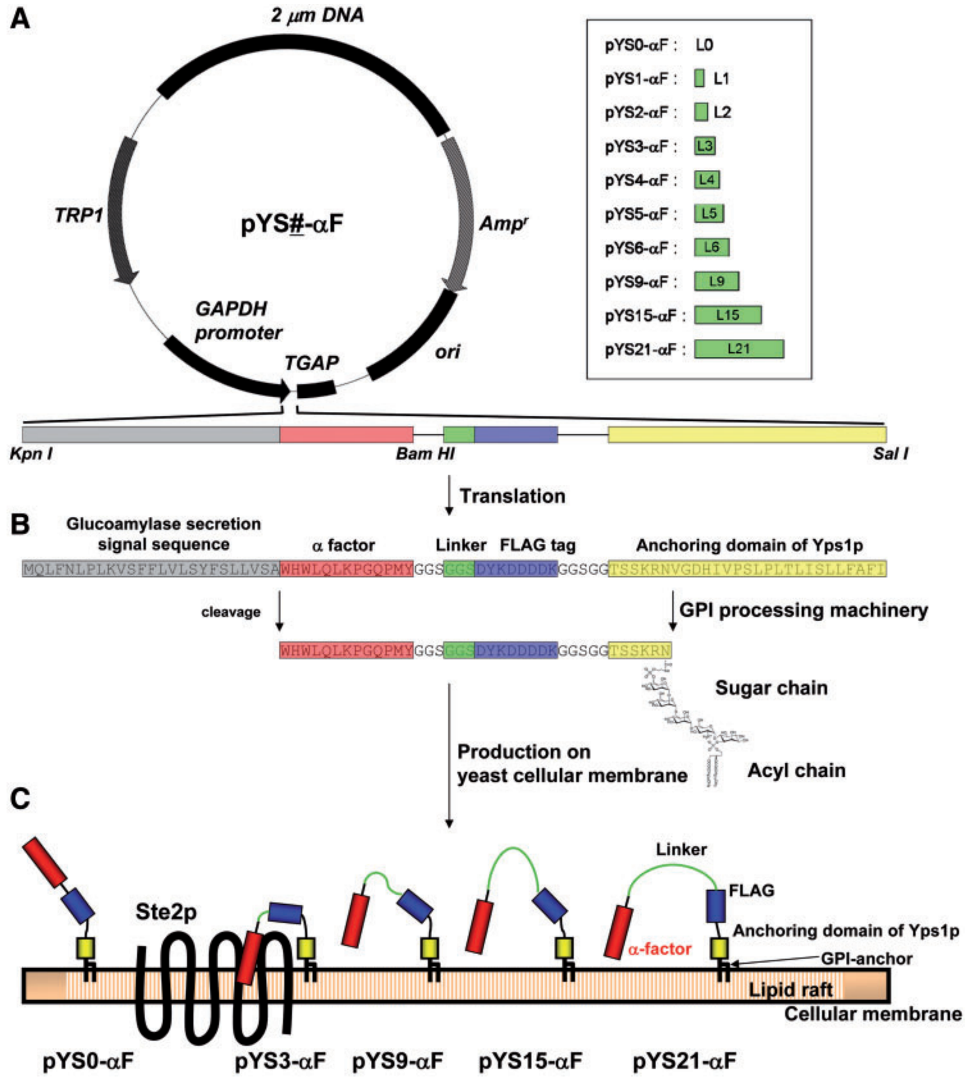

Fig. 2 Construction of α-factor production vectors and display of the produced ligands on the plasma membrane. (A) Diagram of the membrane-displayed ligand plasmid. Underlined number in pYS#-αF varies depending on the linker length. In addition to the negative control not containing the ligand sequences, 10 variants of the linker region were constructed. (B) Functional domains encoded by the membrane-displayed ligand plasmid. After processing by the secretory pathway, the secretion signal and GPI targeting sequences are cleaved and α-factor with a free N-terminus is displayed on plasma membrane by GPI covalently linked to the C-terminus. (C) A schematic representation of membrane-displayed ligands and interaction with receptor.

of the optimal distance between the displayed ligand and GPCR was difficult.

Signalling triggered by membrane-displayed ligand in yeast

We introduced membrane-displayed α-factor plasmids with different linker lengths into WTH-5 strain in Table I. To optimize the signal output, several gene deletions were performed into the negative regulator of Gpa1 activity (*sst2Δ*), the cyclin-dependent kinase inhibitor (*far1Δ*) and aspartyl protease specific for α-factor (*bar1Δ*). Activation of Ste2p was detected by coexpression of pYEX-fet, the high copy number reporter plasmid with *EGFP* controlled by the signal responsive *FUS1* promoter (see 'Materials and Methods' section).

The culture's fluorescence intensity and OD^{600} were measured every 3 h. Maximum fluorescence response per OD unit at 600 nm was observed at 21 h (late-log phase). Among a series of yeast strains possessing one of the membrane-displayed α-factor plasmids, a strain with pYS3-αF exhibited a clear signal (Fig. 3A). This result was further confirmed by fluorescence microscopy and FACS analysis (Fig. 3B). The FACS peak-shift of the yeast strain with pYS3-αF, as well as the clear fluorescence, indicated membrane-triggered signalling. The 'shmoo' formation observed with phase contrast microscopy (Fig. 3B, indicated by arrows), which is the phenotype of the signalling cascade, also supports the activation of Ste2p by membrane-displayed α-factor.

Western blot analysis for confirmation of the membrane-displayed ligand on the plasma membrane

Binding of the peptide ligands on the yeast plasma membrane was confirmed by western blot analysis of the membrane fraction using FLAG tag antibody

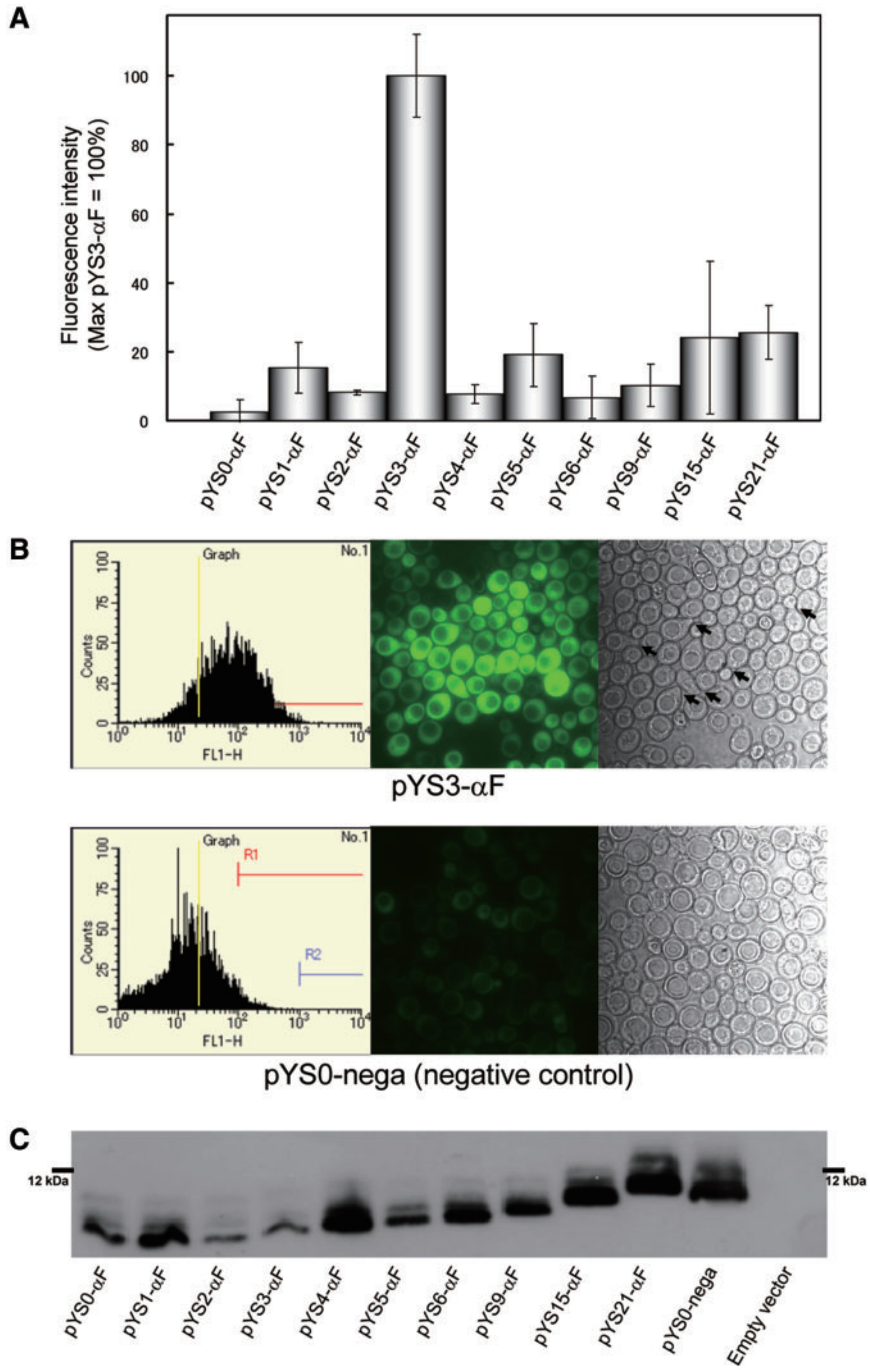

Fig. 3 Activation of the pheromone response pathway in yeast strain with pYS3-αF. (A) Fluorescence intensity of yeast strains transformed with membrane-displayed ligand plasmids. A series of yeast strains with one of the membrane-displayed ligand plasmids was assayed at late-log phase using a 96-well fluorescent plate reader. Yeast cells harbouring pYS3-αF emitted strong fluorescence. Each data point represents data (mean ± standard error of the mean) from 3 independent experiments. (B) FACS analysis and observation under a fluorescence microscope of yeast strain with pYS3-αF. Membrane-displayed α-factor-triggered signalling in yeast strains with pYS3-αF was confirmed by the peak shift in FACS analysis. Clear fluorescence in yeast strain with pYS-αF3 was observed using a fluorescence microscope. 'Shmoo' formation (indicated by arrows) was observed in pYS3-αF cultures, which also supports signalling triggered by membrane-displayed ligand. (C) Western blot analysis of a series of membrane-displayed α-factor proteins. Production of membrane-displayed α-factor on yeast plasma membranes was confirmed by western blot analysis using anti-FLAG tag antibody. The position of 12 kDa of size marker is indicated by black bars on both sides of the blot.

(Fig. 3C). All the recombinant proteins with various linker lengths were present. Only the yeast strain with pYS3-αF activated the signalling pathway, even though all other proteins were produced on yeast plasma membrane.

Comparison of the fluorescence intensity induced by the membrane-displayed α-factor and exogenously added α-factor

We compared the signal strengths resulting from the membrane-bound and exogenously added α-factors

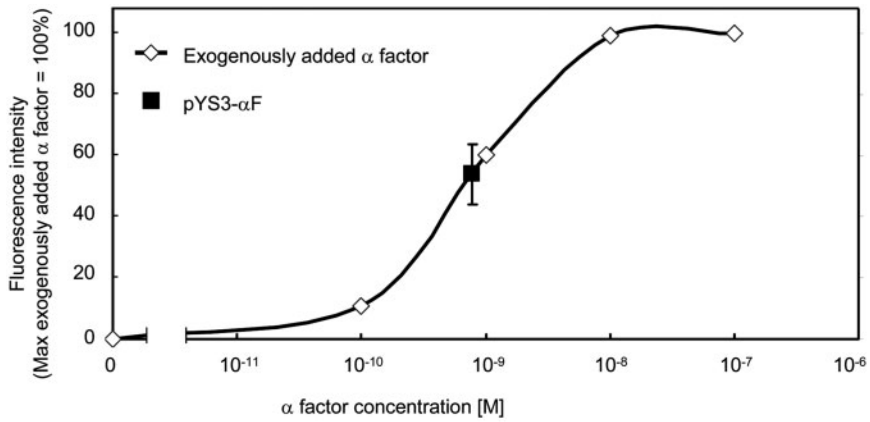

Fig. 4 Comparison of signalling mediated by membrane-displayed or exogenously added α-factor. The signal intensity of the yeast strain transformed by pYS3-αF was equivalent to that of a non-transformed culture treated with 8.6×10^{-10} M α-factor added exogenously and approximates physiological activation.

(Fig. 4). The fluorescence intensity of the ligand-displaying pYS3-αF strain was equivalent to that of strain without ligand-displaying when 8.6×10^{-10} M α-factor was added exogenously.

Discussion

A membrane-displayed peptide ligand system in *S. cerevisiae* can be considered as an extended version of a cell-surface display system (*12*). In the cell-surface display system, the peptide or protein is displayed on cell wall, whereas the peptide in the membrane-displayed ligand system is displayed on plasma membrane. On the other hand, we successfully demonstrated that six amino acids including the ω-site are sufficient for localizing the peptide to the plasma membrane in comparison with the minimum length of 51 amino acid residues reported by others (*11*).

Using the pheromone response pathway as a model system, functional activation of GPCR signalling by the membrane-displayed peptide ligand α-factor was demonstrated by fluorescence emitted by the response of a signal-responsive reporter assay, fluorescence microscopy, FACS analysis and western blotting.

Linker length was crucially important for the activity of the membrane-displayed α-factor, indicating that the topology of the Ste2p-α-factor complex is strictly controlled by a single amino acid residue. Considering that the linker lengths used in the tethered ligands in the experiments using mammalian cells (*19–21*) were different from our present study, our optimal linker length is probably specific for Ste2p and α-factor. This indicates that future studies should determine the optimum linker length for the target receptor.

On the other hand, yeast serve as an ideal host for high-throughput screening of peptides or proteins in a large combinatorial library (*22*, *23*). In spite of the major difference between human and yeast in the heterotrimeric G proteins and the sterol composition of plasma membrane (*24*), many human GPCRs has been functionally produced in yeast using chimeric Gα proteins (*6*). In terms of the sterol composition, the cholesterol-producing yeast was recently reported (*25*), in which the main sterol in yeast is converted from ergosterol to cholesterol by gene deletions and insertions, and thus, the difference in lipidic component of yeast plasma membrane will not be a large hurdle in application to mammalian GPCRs. Collectively, the methodology presented here will provide a new platform for identifying novel peptide ligands for both liganded and orphan mammalian GPCRs.

In conclusion, we have established a membrane-display ligand system in *S. cerevisiae* in which membrane-displayed α-factor linked to the anchoring domain of the GPI-anchored plasma membrane protein Yps1p activates the pheromone response pathway. Important aspects of our study were the discoveries that the length of the linker strongly influenced activity.

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Conflict of interest
None declared.

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