研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
石井 純 田中 勉 荻野 千秋 近藤 昭彦	第3章 「細胞内生体分子群の実測定量解析」 4. フローサイトメトリーとGFPレポーターによるG蛋白質シグナルのシングルセル解析	(監修) 神原秀記 松永是 植田充美	シングルセル解析の最前線	CMC出版	日本	2010	

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Togawa S, Ishii J, Ishikura A, Tanaka T, Ogino C, Kondo A.	Importance of asparagine residues at positions 13 and 26 on the amino-terminal domain of human somatostatin receptor subtype5 in signalling	Journal of Biochemistry	In press		2010
Iguchi Y, Ishii J, Nakayama H, Ishikura A, Izawa K, Tanaka T, Ogino C, Kondo A.	Control of signalling properties of human somatostatin receptor subtype 5 by additional signal sequences on its amino-terminus in yeast	Journal of Biochemistry	In press		2010
Miyachi, Y., Shimizu, N., Ogino, C., Kondo, A.	Selection of DNA aptamers using atomic force microscopy	Nucleic Acids Research	38	e21	2010

シングルセル解析の最前線

第3章　細胞内生体分子群の実測定量解析

4　フローサイトメトリーとGFPレポーターによるG蛋白質シグナルのシングルセル解析

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4　フローサイトメトリーとGFPレポーターによるG蛋白質シグナルのシングルセル解析

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4.1　はじめに

シングルセル解析（1細胞解析）とはその名のとおり，1細胞を解析する技術のことであるが，いったい何のメリットがあるのであろうか？

本節では筆者らがフローサイトメーターとGFPを利用して行った研究例をもとに，シングルセル解析の1つの利点と魅力を紹介する。

4.2　緑色蛍光蛋白質（GFP）とフローサイトメーター

オワンクラゲ*Aequorea victoria*由来の緑色蛍光蛋白質（Green fluorescent protein, GFP）は約27 kDaの分子量からなる蛋白質であり，青色の励起光をあてることにより緑色の蛍光を発する。GFPは単独で発色団を形成し，発色に基質を必要としないため，細胞内で発現させたGFPは細胞を非破壊の状態，かつリアルタイムでその蛍光を観察できる。そのため，GFPはレポーター遺伝子として広く普及し，その発見者である下村脩氏は2008年にノーベル化学賞を受賞した。分子生物学を含む生物系の研究者ならほとんど誰もが一度は耳にしている有名な蛋白質であろう。

フローサイトメトリーとは，微細な粒子を流体中に流し込み，整然と列をなして流れている個々の粒子にレーザー光をあてて反射する光を測定し，その光の強さを電気信号に置き換えて定量化する分析手法のことである[1)]。フローサイトメトリーに用いられる分析装置のことをフローサイトメーターと呼ぶ。粒子として細胞を流すことも可能で，生物分野においても広く使用されている。シース液を一定の速度で流しておき，中央部からサンプル液を注入することで検体が1つ1つ離れた状態で整列して流れるため，その流れにレーザー光を照射することで1細胞ごとの散乱光や蛍光の情報を得ることができる（図1）。なお，個々の細胞の散乱光と蛍光は光電子増倍管（Photo-multiplier tube, PMT）によって高感度に検出される。毎秒数万個の細胞をリアルタイムに分析することができ，各種パラメーターを自動かつ客観的に高分解能で測定できるとい

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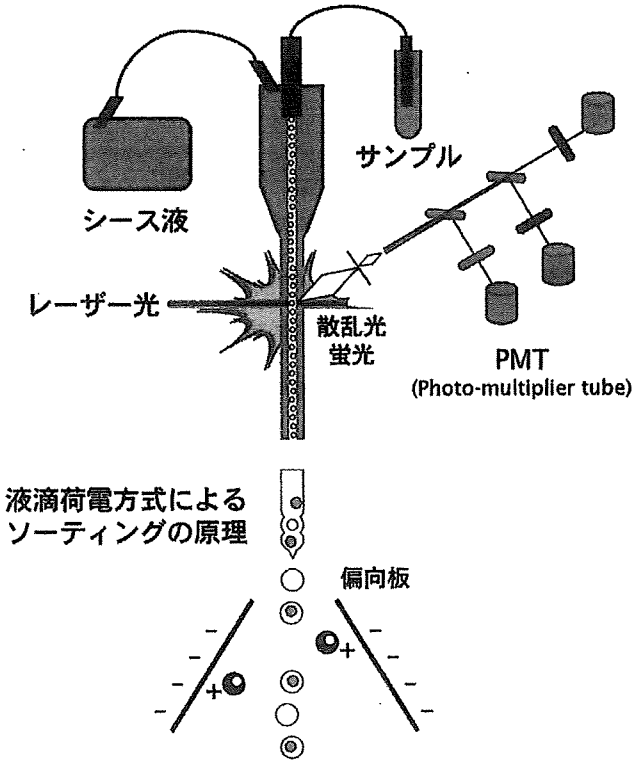


図１　フローサイトメーターのしくみ

う利点を持つ。目的の蛍光を発する細胞を分取することも可能で，分取機能を持つものをセルソーターと呼ぶ。近年のものでは，液滴荷電方式と呼ばれるソーティング（分取）形式を用いたタイプが多い。超音波発生装置による振動でレーザー照射部から少し離れた位置より１細胞ずつを含む液滴に分かれて落下させ，目的の蛍光などを発する細胞の液滴を荷電することで，偏向板により目的の細胞のみを分取できる（図１）。

フローサイトメーターは蛍光標識した抗体などを利用した抗原検出などに広く利用されているが，GFPをレポーターとする組み合わせにおいても威力を発揮する。筆者らの研究例をもとにその一例を見てみよう。

４.３　G蛋白質共役型受容体（GPCR）

G蛋白質共役型受容体（GPCR）は，７回膜貫通型の構造を有する表層蛋白質で細胞外リガンドが結合すると細胞内G蛋白質を介してシグナルを伝達する。GPCRは嗅覚，味覚，視覚あるいは心拍，血圧，神経伝達，細胞増殖など多様な生理機能に関与しており，その調節因子は米食品医薬品局（FDA）の承認医薬品の中でも約30％を占める最主要な創薬ターゲットとなっている[2)]。GPCRはヒトにおいて700～1,000種類程度存在していると見積もられており，リガンド未知のオ

ーファン受容体もまだ多く残されている。これらオーファン受容体を含めたGPCRは，創薬候補となりうる新規リガンドの探索や疾患に関与する作用機序の解明など様々な研究が行われている。

GPCRのアッセイには哺乳動物細胞が使われるのが一般的であるが，筆者らは酵母細胞を宿主とした異種GPCRアッセイ系の開発に取り組んでいる。酵母2ハイブリッド法（Y2H）に代表されるように，酵母細胞は膨大な遺伝子ライブラリを簡便にスクリーニングできるため，ヒトを含む異種GPCRを酵母で発現・アッセイできれば，非常に魅力的なツールとなりうるためである。たとえば，リガンド候補となるペプチドや抗体遺伝子などをライブラリとすることにより，ペプチド創薬あるいは抗体医薬として利用できる分子が取得できるかもしれない。あるいは，GPCRに部位特異的変異かランダム変異を導入したライブラリからスクリーニングを行うことにより大規模な構造解析にも利用できるであろう。酵母は最もシンプルな真核単細胞であり，多様なGPCRおよびシグナル伝達機構を有する高等真核生物に比べて非常にシンプルなGPCRシグナル伝達機構を持つため，アッセイ系を構築するのに非常に適している。実際，ヒトを含む異種GPCRが酵母細胞で機能的に発現し，酵母内G蛋白質を介してフェロモンシグナル伝達経路を活性化することが知られており，アッセイ系の構築に利用されている[3]。

4.4　酵母フェロモンシグナル伝達経路を利用したGFPレポーターによるGPCRアッセイ系

フェロモンシグナル伝達経路は酵母の接合（Mating）機構を誘導する際に必要であることが知られている[4]。酵母a型細胞において，内在性GPCR（Ste2）はリガンドであるフェロモン（α-factor）が結合することにより構造変化を起こし，細胞内の三量体G蛋白質（Gpa1/Ste4/Ste18）を不活性型から活性型（GDP結合型→GTP結合型）に変化させる。活性化されたG蛋白質はGpa1とSte4/Ste18複合体に解離することでシグナルを伝達する（図2）。活性化されたシグナルはMAPキナーゼと呼ばれるカスケードによって増幅され，Far1とSte12という蛋白質をリン酸化する。サイクリン依存性キナーゼ阻害剤としての機能を持つFar1蛋白質はリン酸化されることによりG1期で細胞周期を抑制し，接合準備を整えるために細胞周期を同調する。また，Ste12は転写因子をコードしており，リン酸化されることにより核内に移行しては接合に必要な種々の遺伝子の転写発現を誘導することも知られている。

筆者らはフェロモンシグナルにより転写誘導される*FUS1*という遺伝子に*GFP*遺伝子を融合することで，シグナル伝達に応答して蛍光が発現するシステムを構築した（図2）[5]。Y2Hでも利用されている*lacZ*のような比色による酵素反応用の遺伝子や*HIS3*のような生育セレクション用の遺伝子などによるGPCRアッセイ系はこれまでに開発されていたが，*GFP*をレポーター遺伝子として利用することによりセルソーターによる高速スクリーニングが可能となると考えたので

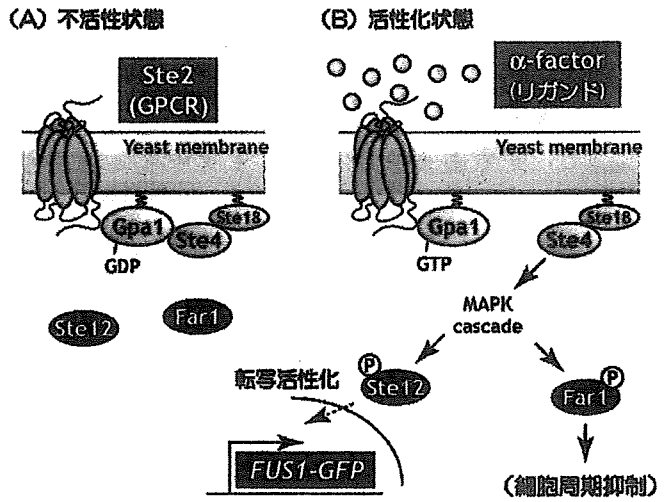


図２　酵母フェロモンシグナル経路を利用したGPCRアッセイ系

ある。酵母を利用したこれまでのシステムでは，細胞周期抑制を誘導するFar1をコードする遺伝子を破壊した株あるいは破壊していない株の両方が利用されており，*GFP*をレポーター遺伝子とした場合，どちらが適しているかを確認する必要があった。そこで，*GFP*レポーター遺伝子を組み込んだ株をもとに*FAR1*遺伝子を破壊した株を構築し，α-factorを添加することでシグナル伝達を引き起こし，*GFP*レポーター発現にどのような影響を与えるかを調べることにした。

4.5　*FAR1*遺伝子破壊株におけるシグナル伝達のフローサイトメトリー解析

*GFP*レポーター遺伝子を組み込んだ細胞にα-factorを添加した場合と添加していない場合でそれぞれ培養し，経時的に細胞をサンプリングしてフローサイトメーターで解析した。解析した細胞の緑色蛍光強度の平均値（１万細胞の平均値）を図３(A)に示す（白色棒グラフ：α-factorを添加していない細胞，黒色棒グラフ：α-factorを添加した細胞)。各細胞においてα-factorを添加することによってシグナルが伝達されてGFPが発現していることが確認され，どの細胞においても12時間後が最も蛍光強度が高くなることが分かった。また，*FAR1*遺伝子を破壊した株が相対的に高い蛍光強度を示すことも明らかとなった。このグラフはシグナル伝達量の平均値を反映したものであり，*GFP*の代わりに*lacZ*などの酵素をレポーター遺伝子として使用した場合でも，よく似た結果が得られると予想される。

一方で，フローサイトメーターで解析した結果の表示形式を変え，緑色蛍光を横軸とし，細胞数を縦軸としたヒストグラムで１万細胞をプロットした結果を図３(B)に示す（色付ヒストグラム：α-factorを添加していない細胞，白抜ヒストグラム：α-factorを添加した細胞)。結果は

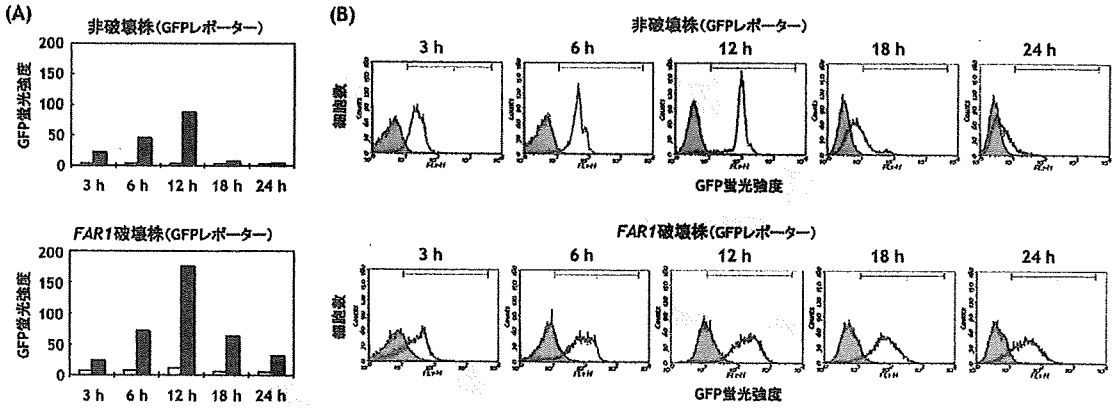


図3　*FAR1*破壊株におけるGFPレポーター発現のフローサイトメトリー解析

上述のグラフと同じものであるが，より細かい情報が見て取れる。α-factorを添加した細胞の12時間のプロットをそれぞれ見ていただくと，*FAR1*を破壊した細胞とそうでない細胞でヒストグラムの形状が明らかに違うことに気づくであろう。たとえば，上段の非破壊株ではGFPの発現量が非常に揃っていることが分かる。これは，*FAR1*非破壊株がシグナル伝達によって細胞周期抑制されて，細胞周期が一致していることを表していると考えられる。逆に下段の*FAR1*破壊株では，細胞周期が抑制されないため，細胞間でのGFPの蛍光強度（発現量）がばらついて一様に分布していると考えられる。このように，１細胞ごとの蛍光強度とその分布を付加的な情報として得ることができることはフローサイトメーターの分解能の高さを物語っており，シングルセル解析ならではの利点と言えるであろう。しかしながら，これらの結果では蛍光強度の高い*FAR1*破壊株を使うべきか，レポーター発現量のばらつきが少ない非破壊株を使用するべきかは人によって判断の異なるところであろう。

4.6　モデルGPCR発現系でのシグナル伝達のフローサイトメトリー解析

次に，異種GPCRを発現することを想定して酵母内在性GPCRが競合発現しないようにするため，Ste2をコードする遺伝子をノックアウトした。また，変異ライブラリなどに応用することも考え，GPCRの発現には環状エピソーム型（ゲノムに組み込まれず複製・保持される）プラスミドを用いることにし，今回はモデルとして異種GPCRの代わりに*STE2*遺伝子を発現させることとした。

図４にリガンドであるα-factorを添加してから，経時的に細胞の緑色蛍光をフローサイトメーターで解析した結果を示す。ゲノム上の*STE2*遺伝子を破壊していない細胞ではほぼ100％の細胞がGFP由来の蛍光を発していた（図４(A)）。しかしながら，ゲノム上の*STE2*遺伝子を破壊し，代わりにエピソーム型プラスミドでSte2を発現する細胞では，時間とともに蛍光を発してい

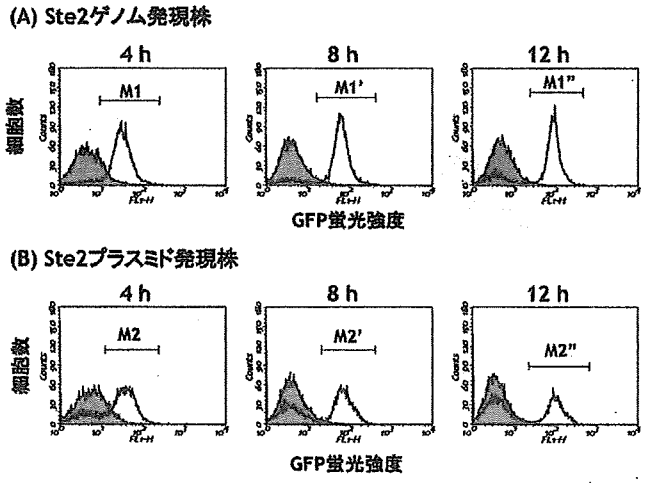
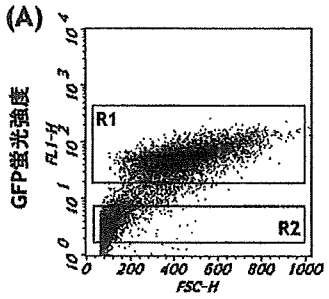


図４　エピソーム型プラスミドによるGPCR発現系におけるシグナル伝達のフローサイトメトリー解析

る細胞の割合が減少し，12時間後にはその割合はおよそ40％程度にまで落ち込んでいることが明らかとなった（図４(B)）。この結果も１細胞ごとの蛍光強度を解析できるフローサイトメーターの大きな利点を表していると言える。たとえば，フローサイトメーター以外の蛍光分析機器を用いた場合，細胞数あたりの蛍光強度は測定できるが，蛍光強度の異なる細胞群の存在やその割合までは通常解析することができない。そのため，単に蛍光強度が弱いという情報しか得られず，蛍光強度の異なる２つの細胞群が存在するということは予想し難いであろう。GFP蛍光を発していない細胞群はシグナルを活性化していないであろうことが予想され，これら２つの細胞群に分かれた原因に*STE2*遺伝子をゲノムからエピソーム型プラスミドに乗せ変えたことが関与していることは明らかである。筆者らは，蛍光を発していない細胞群がSte2を発現していない細胞群であると考え，シグナル伝達に起因したG1期での細胞周期抑制がプラスミドを欠落させているのではないかと仮説を立てた。

４.７　セルソーターによるシグナル活性化細胞群と非活性化細胞群の分取および解析

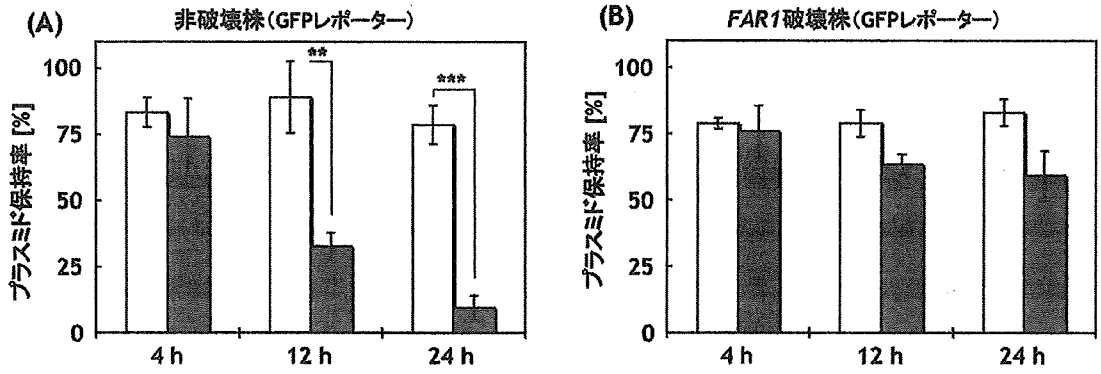
Ste2をエピソーム型プラスミドで発現させる細胞においてα-factor添加後12時間培養し，セルソーターを用いてシグナル活性化細胞群（GFP蛍光強度の高い細胞群）とシグナル非活性化細胞群（GFP蛍光強度の低い細胞群）を別々にソーティングして（図５(A)），それぞれのプラスミド保持率を調べた（図５(B)）。驚くべきことに，蛍光強度の高い細胞群（R1領域）のプラスミド保持率はほぼ100％であったのに対し，蛍光を発していない細胞群（R2領域）のプラスミド保持率はほぼ０％であった（図５(B)）。つまり，エピソーム発現型においてはプラスミドの欠落のためにシグナル伝達が起こらなくなり蛍光を失っていることが証明された。この結果は１細胞ご



(B)

プラスミド保持率	
R1 領域	R2 領域
97.4%	0.6%

図5　セルソーターによるシグナル活性化細胞群と非活性化細胞群のソーティングおよびプラスミド保持率の解析



(C)

時間 (h)	非破壊株	*FAR1*破壊株
4	88.3%	96.1%
12	37.2%	81.0%
24	11.6%	72.0%

図6　*FAR1*遺伝子破壊がプラスミド保持率へ与える効果

とに蛍光を解析できることに加え，目的細胞を分取できるセルソーターの大きな利点を証明している。また，筆者らの考えるGPCRアッセイ系でのセルソーターによる高速スクリーニングの実現可能性も示唆している。

これらフローサイトメーターを利用した解析結果から，シグナル伝達によるG1期での細胞周期抑制がプラスミドを欠落させているという仮説を確かめるため，*FAR1*破壊株と非破壊株にα-factorを添加後，経時的にサンプリングしてプラスミド保持率を調べた（図6(A)~(C)）。*FAR1*を破壊していない株では時間とともに明らかにプラスミド保持率が激減したのに対し，*FAR1*破壊株ではプラスミドの保持率が大幅に改善される結果となった（図6(C)）。このことから筆者らの仮説の妥当性が証明された。

遺伝子ライブラリからのスクリーニングを考えた場合，プラスミドが脱落するということはア

ッセイが不可能になる細胞が増えるというだけでなく，スクリーニングしたい遺伝子を回収できなくなるという致命的な問題につながることが予期されるため，*FAR1*遺伝子の破壊によってプラスミド保持率が維持されるという結果は蛍光レポーターを用いたGPCRアッセイ系によるハイスループットスクリーニング系を構築する上において有益な情報となるであろう。

*FAR1*遺伝子を破壊していない株がエピソーム型プラスミドを用いたGPCR発現系において，フローサイトメーターとGFPを用いなくてもレポーターの発現が悪くなることもしくはプラスミドの保持率に大きく影響することを予想できる研究者はいるかもしれない。しかしながら，筆者らが行ったようにシグナルを活性化している細胞群と活性化していない細胞群を分離して別々に解析することは，おそらくフローサイトメーターとGFPを用いた系以外ではほとんど不可能であったと自負しており，そこにシングルセル解析の利点と魅力が隠されていると思っている。

4.8　おわりに

フローサイトメーターおよびGFPが世に広く普及するようになってからもうすでに久しく年月を重ねており，これらの有用性は言うまでもない。フローサイトメーターとGFPの組み合わせによるシングルセル解析，ありきたりの組み合わせのように感じられるかもしれないが，まだまだ応用できる分野は広く存在すると思われる。筆者らは今回紹介した研究以外にもフローサイトメーターとGFPを利用して酵母用発現ベクターを簡便に評価できるシステムも報告しているので，もし興味のある方は御一読いただけると甚幸である[6]。今回紹介したアプローチはあくまでシングルセル解析のほんの一例であり，なじみの薄いテーマであったかもしれないが，少しでもシングルセル解析の利点・魅力が伝われればと切に願っている。

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Importance of asparagine residues at positions 13 and 26 on the amino-terminal domain of human somatostatin receptor subtype-5 in signalling

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N-linked oligosaccharides or asparagine residues are often involved in G protein-coupled receptor functions. Focusing on Asn13 and Asn 26 positioned on N-linked glycosylation motifs in the amino-terminal domain of human somatostatin receptor subtype-5 (hSSTR5), we performed site-directed mutagenesis and evaluated the mutants by using yeast cells as the host strain. This is because analysing the complicated signalling in mammalian cell lines is simplified by the utilization of the monopolistic pheromone signalling pathway in yeast. Western blot analysis and confocal laser scanning microscope observation showed that Asn13 and/or Asn26 mutations had no effects on cell-surface expression of hSSTR5 in yeast. By using an engineered yeast strain of *Saccharomyces cerevisiae*, which induces the expression of the green fluorescent protein (*GFP*) reporter gene in response to the agonist-specific signal transduction, it was demonstrated that a single mutation of two asparagine residues attenuated the somatostatin-specific signalling levels, and the double mutant significantly lost the signalling ability. These results clearly show the importance of these asparagine residues in the agonist-specific signalling of hSSTR5, although it was not enough to identify the consequence of oligosaccharides.

Keywords: G protein-coupled receptor/green fluorescent protein/mutagenesis/somatostatin receptor/yeast.

Abbreviations: FDA, Food and Drug Administration; GFP, green fluorescent protein; *EGFP*, enhanced green fluorescent protein; G-proteins, guanine nucleotide binding proteins; GPCR, G protein-coupled receptor; SSTR, somatostatin receptor; hSSTR5, human somatostatin receptor subtype-5; YPD, yeast extract–peptone–dextrose; SD, synthetic dextrose; *PGK5′*, *PGK1* promoter; *PGK3′*, *PGK1* terminator; 5-FOA, 5-fluoroorotic acid; PMSF, phenylmethylsulphonyl fluoride; 2-ME, 2-mercaptoethanol.

G protein-coupled receptors (GPCRs) constitute the largest family of transmembrane receptors and are considered attractive for pharmaceutical and therapeutic targets in the field of drug discovery. Currently, nearly 30% of Food and Drug Administration (FDA)-approved drugs are modulators of GPCR function (*1*). These receptors transduce the external stimuli and the binding of ligands into intracellular signals, which underlie a variety of biological processes, via the intracellular guanine nucleotide-binding proteins (G-proteins) (*2*, *3*). The heterotrimeric G-proteins universally comprise the Gα, Gβ and Gγ-subunits and are classified into various classes in humans—many GPCRs, by using complex mechanisms, activate the various signalling pathways through these peripheral G-proteins.

The budding yeast *Saccharomyces cerevisiae* is a familiar host cell system to study GPCRs because it simplifies the analyses of the complicated signalling in mammalian cell lines (*4*). The yeast possesses the uncompetitive and monopolistic G-protein signalling pathway (pheromone-signalling pathway), and it has successfully coupled a variety of human GPCRs to the yeast pheromone signalling pathway via the sole endogenous Gα-subunit (*5*). Therefore, the ligand-specific activation of human GPCRs is detectable by several types of reporter genes, which are expressed when the pheromone responsible promoters are utilized in responding to the ligand-specific stimulation via pheromone signalling (*6*, *7*). Several mutagenesis studies investigating the important domains on human GPCRs in yeasts have demonstrated that the yeast cells are convenient tools to determine the critical amino-acid residues for their functions (*8–10*).

Somatostatin, a cyclic neuropeptide known as a growth hormone release-inhibiting factor, is a natural ligand of somatostatin receptors. Five subtypes of somatostatin receptors have been identified (SSTR1–SSTR5) (*11*, *12*), and they are noticeable for therapeutic targets of acromegaly, Cushing's disease and Alzheimer's disease (*13–15*). They are widely expressed in several organs with tissue-specific distribution patterns; SSTR2 and SSTR5, in particular, predominantly regulate growth-hormone secretion in acromegaly patients (*16*). Therefore, information on the structural differences of these receptors might be attractive for subtype-specific drug designs or the presumption of significant mutations involved in these diseases.

GPCRs are typical heptahelical receptors, and they are composed of an extracellular amino-terminus, an

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intracellular carboxy-terminus and seven transmembrane bundles, which are connected by three intracellular loops and three extracellular loops. A wide variety of motifs and amino-acid residues in these domains characterize the structural roles of GPCRs with receptor-specific differentiations. The consensus sequences for N-linked glycosylation (Asn-X-Thr or Asn-X-Ser) in which oligosaccharides could bind to the asparagine residues (*17*) are found in many GPCRs and are shared by almost all eukaryotes, including yeast cells (*18*). The significance of these asparagine residues has been demonstrated in various receptors and they seem to play several important roles, for example, in ligand affinity, and plasma membrane localization and signalling activation, although the role varies with (i) the receptor type addressing the functions, (ii) the N-linked oligosaccharides, (iii) or the side chains of asparagine residues themselves (*19–22*). In the case of human SSTRs, the extracellular amino-terminal domain of all subtypes (SSTR1–5) certainly includes one or more N-glycosylation sites (*23*). Mutations of Asn18 and Asn31 residues on the N-glycosylation sites in rat SSTR3 affect somatostatin ligand binding and the inhibition of adenylyl cyclase activity in signal transduction (*24*). However, there are no reports on the mutagenesis of human SSTRs on these sites. Therefore, we focused on the 13th and 26th asparagine residues forming the N-glycosylation motifs on the amino-terminal domain of human SSTR5 (hSSTR5) because of the lower affinity of available therapeutic medicine, octreotide and lanreotide to SSTR5 than to SSTR2 (*15*).

In this study, we performed site-directed mutagenesis of asparagine residues on the two N-linked glycosylation motifs in the amino-terminal domain of hSSTR5. The alanine residues were substituted for Asn13 and Asn26 residues in the amino-terminal domain of hSSTR5, and the mutants were analysed for expression, localization and signalling activity. In order to quantitatively estimate the signalling activity of the receptors, the yeast *S. cerevisiae* was selected as the host, and the engineered yeast strain was constructed to induce the expression of the *GFP* reporter gene responding to ligand binding (*25*). Through this work, we show that the double substitution of asparagine residues on the N-glycosylation motifs in the amino-terminus of hSSTR5 significantly decreases the signalling activity, and we also show the availability of a yeast-based fluorescent assay for GPCR signalling analysis.

Materials and methods

Yeast strain

The yeast strain IMFD-70 (*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 far1Δ sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::EGFP his3Δ::*P_{FIG1}*-EGFP*) was used in this assay. This strain was derived from BY4741 and was constructed as follows. Each linear DNA fragment was introduced into yeasts using the lithium acetate method (*26*). In accordance with the marker recycling method, the *URA3* marker was eliminated by homologous recombination, using counter selection in each transformation step (*27*).

The plasmid used for substituting the fluorescence reporter gene for the *FIG1* gene on the yeast chromosome was constructed as follows: DNA fragments encoding the *FIG1* promoter (300 bp) and *FIG1* terminator (300 bp) were amplified by PCR from BY4741 genomic DNA with the following oligonucleotide pairs: o1 and o2; and o3 and o4. The amplified fragments were, respectively, digested with *Eco*RI/*Bam*HI and *Bam*HI/*Sph*I and ligated at the *Eco*RI/*Sph*I sites on pCR4 Blunt-TOPO vector (Invitrogen, Carlsbad, CA). The resultant plasmid was named pFIG1pt. A DNA fragment encoding the enhanced green fluorescent protein (*EGFP*) gene was amplified from pEGFP (Takara Bio, Shiga, Japan) by PCR with the oligonucleotides o5 and o6. The amplified fragment was digested with *Bgl*II/*Bam*HI and ligated at the *Bam*HI site on pFIG1pt. The resultant plasmid was named pFIG1GF. A DNA fragment encoding the *URA3* selectable marker (with 40 nucleotides from the 5′-side of the *FIG1* terminator at the 5′-end) was amplified by PCR from pRS426 (American Type Culture Collection, Manassas, VA) with oligonucleotides o7 and o8. The amplified fragment was digested with *Bam*HI and ligated at the same site on pFIG1GF. The resultant plasmid was named pMR-FIG1GF. A DNA fragment encoding the *FIG1* promoter (450 bp) and a DNA fragment encoding the *EGFP* gene, *URA3* selectable marker, and *FIG1* terminator were amplified by PCR from BY4741 genomic DNA and pMR-FIG1GF with the following oligonucleotide pairs: o9 and o10; and o4 and o5. The amplified fragments were digested with *Eco*RI/*Bam*HI and *Bgl*II/*Sph*I and ligated at the *Eco*RI/*Sph*I sites on the pUC119 vector (Takara Bio). The resultant plasmid was named pFIG450GF. Two DNA fragments encoding the homologous sequence of the *HIS3* locus were amplified from the BY4741 genomic DNA with the following oligonucleotide pairs: o11 and o12; and o13 and o14, and a DNA fragment containing the *FIG1* promoter, *EGFP* gene, 40 nucleotides (of *FIG1* terminator), the *URA3* selectable marker and *FIG1* terminator was amplified from pFIG450GF with oligonucleotides o15 and o16. A DNA fragment was amplified by overlap PCR, using three amplified fragments as templates with oligonucleotides o11 and o14. The amplified fragment was digested with *Eco*RI/*Sph*I and ligated at the same sites on the pUC19 vector (Takara Bio). The resultant plasmid was named pHIS3-FIG1GF.

A DNA fragment containing the *FIG1* promoter, *EGFP* gene, 40 nucleotides (of *FIG1* terminator), *URA3* selectable marker and *FIG1* terminator was amplified by PCR from pMR-FIG1GF with oligonucleotides o17 and o18, and the amplified fragment was introduced into IM-50 (*25*). After confirming correct integration, the *URA3* marker was eliminated by homologous recombination using counter selection with 5-fluoroorotic acid (5-FOA; Fluorochem, Derbyshire, UK). The strain substituted with the *EGFP* gene for the *FIG1* gene was designated IMF-50. A DNA fragment containing a homologous sequence of the *HIS3* terminator region, *FIG1* promoter, *EGFP* gene, 40 nucleotides (of *FIG1* terminator), *URA3* selectable marker, *FIG1* terminator and a homologous sequence of the *HIS3* promoter region were prepared by digestion of pHIS3-FIG1GF with *Eco*RI/*Sph*I, and the fragment was introduced into IMF-50. After confirming correct integration, the *URA3* marker was eliminated by counter selection with 5-FOA, and the strain integrated with P_{FIG1}-*EGFP*-T_{FIG1} into the *HIS3* locus was designated IMFD-50.

A DNA fragment encoding the *URA3* selectable marker (containing a homologous sequence to delete the *FAR1* gene and one to eliminate the *URA3* marker) was amplified from pRS426 with oligonucleotides o19 and o20, and a DNA fragment encoding the *FAR1* terminator region was amplified from BY4741 genomic DNA with oligonucleotides o21 and o22. A DNA fragment was amplified by overlap PCR, using two amplified fragments as templates with oligonucleotides o19 and o22, and the amplified fragment was introduced into IMFD-50. After confirming correct integration, the *URA3* marker was eliminated by counter selection with 5-FOA. The constructed strain, whose *FAR1* gene was disrupted, was designated IMFD-70.

Media

The strain was cultured in a YPD medium [containing 1% yeast extract (Nacalai Tesque, Kyoto, Japan), 2% peptone and 2% glucose; w/v] or an SD medium [containing 0.67% yeast nitrogen base without amino acids (Becton, Dickinson and Co., Franklin Lakes, NJ) and 2% glucose; w/v]. The SD medium was supplemented with appropriate amino acids depending on the desired selectable marker (20 mg/l histidine, 60 mg/l leucine and 20 mg/l methionine or 20 mg/l uracil and without amino acids for auxotrophic selectivity). In accordance with the signalling assay, the SD medium was adjusted to pH 7.1 with the MOPSO buffer to attain a final concentration of

200 mM and was named SDM71. For solid media, 2% agar was added to the media described above.

Plasmid construction and transformation

The plasmids used for the expression of hSSTR5 were constructed as follows. A DNA fragment encoding the 2 μ origin was prepared by digesting pRS402 + 2 μm (*28*) with *Aat*II and was ligated with the same site into pRS401 (American Type Culture Collection), resulting in pRS401 + 2 μm. The fragment containing the *PGK1* promoter (*PGK5'*), multiple cloning site (*MCS*) and *PGK1* terminator (*PGK3'*) was prepared by digesting pTA2-PGK (*28*) with the *Xho*I and *Not*I sites, and then ligated with the same sites into the pRS401 + 2 μm, producing pGK421. A DNA fragment encoding the *hSSTR5* gene containing the HA tag at the carboxy terminus was amplified from the human brain cDNA library (Invitrogen) with oligonucleotides o23 and o24. The amplified fragment was inserted into pBlueScript II KS (+) vector (Stratagene, La Jolla, CA) at the *Eco*RV site. The resultant plasmid was named pBlue-SSTR5-HA. The plasmid was digested with *Nhe*I/*Bgl*II and ligated into pGK421. The resultant plasmid was named pGK-SSTR5-HA.

Site-directed mutagenesis was carried out using a Quikchange Site-directed mutagenesis kit (Stratagene) according to the manufacturers' protocol. The plasmid pBlue-SSTR5-HA was used as a template with the following primers: o25 and o26 for N13A mutation and o27 and o28 for N26A mutation. The resultant plasmids were named pBlue-SSTR5-N13A and pBlue-SSTR5-N26A, respectively. Using the plasmid pBlue-SSTR5-N13A as the template, site-directed mutagenesis was carried out with the primers o27 and o28. The resultant plasmid was named pBlue-SSTR5-N13/26A. The DNA fragments encoding the mutated *hSSTR5* gene was digested with *Nhe*I/*Bgl*II and ligated into pGK421. The resultant plasmids were named pGK-SSTR5-N13A, pGK-SSTR5-N26A and pGK-SSTR5-N13/26A.

A DNA fragment encoding the *EGFP* gene was amplified by PCR from pEGFP (Takara Bio) with oligonucleotides o29 and o30. The amplified fragment was digested with *Bam*HI/*Bgl*II and ligated at the same sites on pGK421. The resultant plasmid was named pGK421-C-GFP. The DNA fragments encoding the wild-type or mutated *hSSTR5* were amplified by PCR from pBlue-SSTR5-HA, N13A, N26A and N13/26A with oligonucleotides o31 and o32. The amplified fragment was digested with *Nhe*I/*Bgl*II and ligated at the *Nhe*I/*Bam*HI sites on pGK421-C-GFP. The resultant plasmids were named pSSTR5-HA, pSSTR5-N13A-GF, pSSTR5-N26A-GF and pSSTR5-N13/26A-GF. Transformation of IMFD-70 by each plasmid was carried out by the lithium acetate method (*26*).

Cultivation of yeast cell-expressing hSSTR5 mutants

The yeast strain IMFD-70 transformed with the wild-type or mutated hSSTR5 expression plasmid was grown in appropriate auxotrophic SD media at 30°C overnight. The cells were inoculated into appropriate auxotrophic SD media to yield an initial optical density of 0.03 at 600 nm, and the cells were grown at 30°C for 18 h.

Western blot analysis

The cultured cells were suspended in 10 mM Tris–HCl (pH 7.8) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) to yield an optical density of 50 at 600 nm, and 200 μl of the cell suspensions was disrupted using multi-beads shocker (Yasui Kikai, Osaka, Japan) with 0.5-mm glass beads. The cell lysate was centrifuged at 1,000*g* for 5 min, and the pellet was washed three times with 10 mM Tris–HCl (pH 7.8) containing 1 mM PMSF. The pellet was re-suspended with 200 μl of buffer [50 mM Tris–HCl (pH 7.8), 2% (w/v) SDS, 100 mM EDTA, 40 mM 2-mercaptoethanol (2-ME)], and the suspension was boiled at 95°C for 5 min and then centrifuged at 10,000*g* for 5 min. The supernatant was collected; an equivalent amount of sample buffer [125 mM Tris–HCl (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-ME, 0.1 mg/ml BPB] and the suspension was incubated at room temperature. In this step, half the supernatant was treated with glycosidases Endo H_f or PNGase F (New England Biolabs, Ipswich, MA) according to the manufacturers' procedure before adding the sample buffer. Proteins were separated on a 10% SDS–polyacrylamide gel by electrophoresis and then transferred to a PVDF membrane (Immobilon-FL; Millipore, Billerica, MA) followed by western blot analysis. Rabbit anti-HA antibody (Bethyl Laboratories, Montgomery, TX) was primarily used at a dilution of 1:5,000 in TBST [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% (v/v) Tween-20], and a goat anti-Rabbit IgG horse radish peroxidase (HRP) conjugate (American Qualex, San Clemente, CA) was used at a dilution of 1:5,000 in TBST. A chemiluminescent reaction was performed with Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK), and the signal was detected using the lumino-image analyzer LAS-1000mini system (Fujifilm, Tokyo, Japan).

Fluorescence imaging by confocal laser scanning microscopy

The cultured cells were washed and suspended in distilled water to yield an optical density of 40 at 600 nm. The cell suspensions were observed with a confocal laser scanning microscope (LSM 5 PASCAL; Carl Zeiss, Oberkochen, Germany). Fluorescence images were acquired using the 488 nm line of an argon laser for excitation and a 505-nm band pass filter for emission.

Signalling assay and flow cytometry analysis

The cultured cells were washed and suspended in distilled water to yield an optical density of 10 at 600 nm. The cell suspensions and somatostatin (Calbiochem, Darmstadt, Germany) (added to yield final concentrations of 5, 10, 25, 50, 75, 100, 250, 500 and 750 nM, and 1, 2.5, 5 and 10 μM) were dispensed into 96-well cluster dishes containing SDM71 media. The plate was incubated at 30°C with shaking at 150 rpm for 4 h. The GFP expression levels in response to each signal transduction were analysed by a flow cytometer (FACSCanto II, Becton Dickinson and Co.). A total of 10,000 cells was analysed for each transformant. The green fluorescence signal was exited with a 488-nm blue laser and collected through a 530/30-nm band-pass filter, and the intensity was defined as the GFP-A mean of 10,000 cells. Assays were performed using three independent transformants.

Results and discussion

Expression of wild-type hSSTR5 and alanine-substituted mutants for two asparagine residues on the amino-terminus in S. cerevisiae

To investigate the importance of the asparagine residues at the 13th and 26th positions on the amino-terminal domains in hSSTR5, two asparagine residues were replaced with alanine residues. The expression plasmids for wild-type hSSTR5 and three resulting mutant receptors that have single (N13A or N26A) and a double substitution (N13/26A) were introduced into an IMFD-70 yeast strain.

Western blot analysis was initially performed with SDS-extracted fractions to examine the expression of the receptor proteins in yeast cells by using the anti-HA antibody. As shown in Fig. 1A, the major specific bands of the wild-type and mutant receptors were confirmed; equal-sized bands were never detected in the case of yeast cells harbouring the mock vector, verifying that hSSTR5 and its mutants were expressed in the yeast cells. The wild-type receptor and single mutant receptors (N13A and N26A) were slightly displayed as subsequent specific bands just above the main bands, while the double mutant receptor (N13/26A) never displayed a similar band. Although the non-specific second minor band from the top showed overlaps, it appeared as the thick HA-specific band of the wild-type receptor when compared with the other mutant receptors. These receptor-specific bands with minor amounts but certainly higher molecular weights were probably considered as the glycosylated receptors on the asparagine residues at the 13th or 26th position.

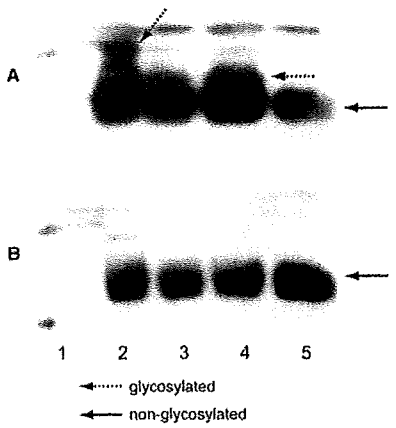

Fig. 1 Western blot analysis of hSSTR5s expressed in yeast. Membrane preparations of hSSTR5s in yeast transformants were analysed by SDS/PAGE (10% gel) and immunoblotted with the HA monoclonal antibody (**A**). The same samples were treated with endoglycosidase (Endo H_f) (**B**). Lane 1: mock, lane 2: wild-type, lane 3: N13A single mutant, lane 4: N26A single mutant, lane 5: N13A and N26A double mutant.

To verify the glycosylation of the receptor proteins in the yeast cells, the same SDS-extracted fractions were treated with endoglycosidase (Endo H_f). Western blot analysis showed that all bands of presumed glycosylated-receptors disappeared (Fig. 1B). Another glycosidase treatment with PNGase F also exhibited similar results (data not shown). The results of these western blot analyses suggest that the wild-type hSSTR5 and the mutant receptors were surely expressed in yeast and were barely glycosylated at the 13th and 26th asparagine residues on the motifs with a slight degree of modification efficiency.

Observation of localization of hSSTR5 mutants fused with a GFP tag by using confocal laser scanning microscopy

To investigate the localization of the receptors in yeast cells, a GFP tag protein was fused to the wild-type hSSTR5 and mutant receptors at the carboxy terminus. Figure 2 shows the confocal laser scanning microscope images of the yeast cells harbouring the GFP-fused receptor expression plasmids. Green fluorescence was localized to the cell membrane at some levels in yeast cells expressing the wild-type hSSTR5-GFP fusion protein. All three mutant hSSTR5-GFP fusion proteins were also reasonably localized on the surface membranes in the yeast cells. These results indicated that the mutation of Asn13 and Asn26 residues did not affect the localization on the yeast cell surface.

Evaluation of somatostatin-specific signalling ability of hSSTR5 mutants, using yeast-based GFP fluorescent reporter gene assay by flow cytometry

The IMFD-70 yeast strain constructed in this study was designed to robustly induce the expression of the *GFP* reporter gene under the control of the pheromone

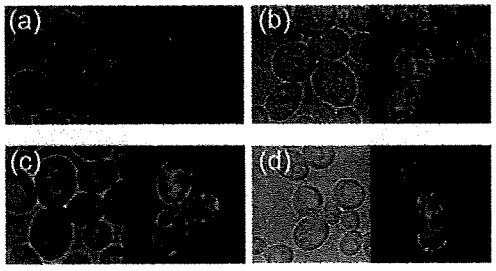

Fig. 2 Localization of wild-type and mutated hSSTR5s. Wild-type and mutated hSSTR5s were fused with GFP at each carboxy terminus. Yeast cells expressing the GFP-fused hSSTR5s were grown in SD-selectable media for 18 h. The cell suspensions were observed with a confocal laser scanning microscope. (**A**) wild-type, (**B**) N13A, (**C**) N26A, (**D**) N13A and N26A.

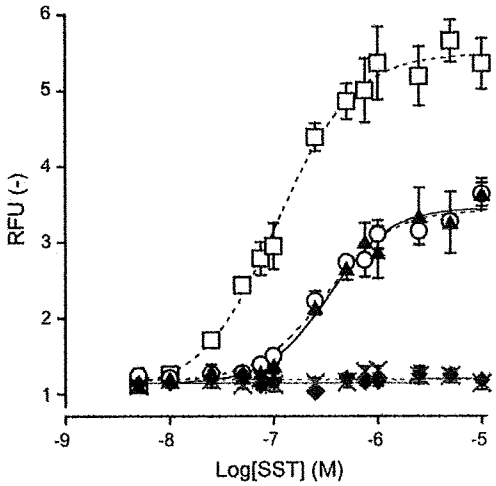

Fig. 3 Dose–response curves of signalling levels of wild-type and mutated hSSTR5s. All transformants were grown in SD selectable media for 18 h. The cell suspensions and each concentration of somatostatin were added into SDM71 media and incubated at 30°C for 4 h. The mean value of the green fluorescence signal of 10,000 cells analysed on flow cytometer is displayed. Data points represent the mean ± SEM obtained from three different experiments and are normalized to the fluorescence measure in the absence of ligand. multi: Mock, open square: wild-type, filled triangle: N13A, open circle: N26A, filled rhombus: N13A and N26A.

responsible *FIG1* promoter in response to ligand-specific signalling through a human receptor. Therefore, the ligand-specific signalling activation levels of human GPCRs could be easily and instantly measured with non-destructive cells on a flow cytometer. The yeast strain IMFD-70 was used for quantitative evaluation of the signalling ability of hSSTR5 responding to the somatostatin stimulation.

To evaluate the somatostatin-specific signalling ability of the wild-type hSSTR5 and its mutants, the yeast-based *GFP* reporter gene assay was carried out. Figure 3 shows the dose–response curves for somatostatin-specific signalling in IMFD-70 cells expressing wild-type hSSTR5 and mutant receptors

Table 1. Oligonucleotides.

Oligonucleotide	Sequence
o1	5′-GGGGGAATTCTACAAAAATTATAACATTTT-3′
o2	5′-CCCCGGATCCTTTTTTTTTTTTTTTTTGT-3′
o3	5′-GGGGGGATCCTTTTATCCTCAAATAAACAT-3′
o4	5′-CCCCGCATGCATAACATTAGTATTTATAAA-3′
o5	5′-CTTTAGATCTATGGTGAGCAAGGGCGAGGA-3′
o6	5′-CCCCGGATCCTTACTTGTACAGCTCGTCCA-3′
o7	5′-GGGGGGATCCTTTTATCCTCAAATAAACATAT AAGTTTTGAGCGGATATTTTTTTTGTTCTTTTTT TTGATTCCGGTTTC-3′
o8	5′-CCCCGGATCCGGGTAATAACTGATATAATT-3′
o9	5′-GGGAGAATTCATCACCCTGCATTGCCTCTT-3′
o10	5′-CCCCGGATCCTTTTTTTTTTTTTTTTTGTTTG TTTGTTTGTTTGTTTAC3′
o11	5′-CCCCGAATTCCGAGTTCAAGAGAAAAAAAA-3′
o12	5′-GCAATGCAGGGTGATTGACACCGATTATTT-3′
o13	5′-AAATACTAATGTTATCTTTGCCTTCGTTTA-3′
o14	5′-GAAAGCATGCTCTTGGCCTCCTCTAGTACA-3′
o15	5′-AAATAATCGGTGTCAATCACCCTGCATTGC-3′
o16	5′-TAAACGAAGGCAAAGATAACATTAGTATTT-3′
o17	5′-TAAGATTATGATGGTTTCATGTATGTGTCA-3′
o18	5′-TTAGTCGCTCATCAAGGTGACAGTAAATAA-3′
o19	5′-CCACTGGAAAGCTTCGTGGGCGTAAGAAGG CAATCTATTATAGTTCGGGAATCGAGGCCCG TATTTCGAGGCTTTTGCTTTTTTTTGTTCTTTT TTTTGA-3′
o20	5-′AAGCAAAAGCCTCGAAATACGGGCCTCGAT TCCCGAACTAGGGTAATAACTGATATAATT-3′
o21	5′-GTATTTCGAGGCTTTTGCTTTTCCTTTTTT-3′
o22	5′-GCATATATGACGAGATTTAATTATCGCCAA-3′
o23	5′-TTTTGCTAGCATGGAGCCCCTGTT-3′
o24	5′-TTGGAGATCTTCAAGCGTAATCTGGAACATC GTATGGGTACAGCTTGCTGGTCTG-3′
o25	5′-GCCTCCACGCCCAGCTGGGCCGCCTCCTCCCCGGGGGCT-3′
o26	5′-AGCCCCCGGGGAGGAGGCGGCCCAGCTGGGCGTGGAGGC-3′
o27	5′-GCCTCTGGAGGCGGTGACGCCAGGACGCTGGTGGGGCCG-3′
o28	5′-CGGCCCCACCAGCGTCCTGGCGTCACCGCCTCCAGAGGC-3′
o29	5′-TTTTGGATCCGTGAGCAAGGGCGAGGAGCT-3′
o30	5′-GGGGAGATCTTTACTTGTACAGCTCGTCCA-3′
o31	5′-TTTTGCTAGCATGGAGCCCCTGTTCCCAGC-3′
o32	5′-GGGAAGATCTCAGCTTGCTGGTCTGCATAA-3′

Underlined sequences indicate complementary pairs for overlap PCR.

(N13A, N26A and N13/26A). Wild-type hSSTR5 displayed obvious GFP fluorescence depending on various somatostatin concentrations in the yeast cells, and the dose-response of hSSTR5 signalling responding to the somatostatin-specific activation was thus confirmed. On the other hand, the single mutations in hSSTR5 (N13A and N26A) considerably decreased the dose–responses of somatostatin-specific signalling. Moreover, the double mutant of hSSTR5 (N13/26A) significantly and completely lost the ability to respond to somatostatin-specific signalling. In fact, the half maximal effective concentration (EC_{50}) values of the cells expressing the single mutant receptors were ~3-fold higher than those of the wild-type receptor-expressing cells (wild-type, 109 ± 11 nM; N13A, 335 ± 44 nM and N26A, 312 ± 44 nM), while the double mutant-expressing cells were incapable (N13/26A). In addition, the single substitutions of glutamine residues for asparagine residues (N13Q and N26Q) substantially decreased, and the double substitutions of glutamate, glutamic acid, lysine and phenylalanine residues for both asparagine residues (N13/26Q, N13/26E, N13/26K and N13/26F) did not exhibit the signalling activity of hSSTR5 either (data not shown). These results clearly show that the asparagine residues at the 13th and 26th positions in the amino-terminus of hSSTR5 are important for receptor function, although the properties of amino-acid side chain (*i.e.* charge or size) do not affect ligand binding. Hence, it might be that the oligosaccharides on amino-terminal domain of hSSTR5 concern the binding of somatostatin to receptor.

Finally, two asparagine residues of human SSTR5 in the amino-terminus exhibited a similar function to that of rat SSTR3 (*24*). The fact that hSSTR5 was barely N-glycosylated (Fig. 1) and high mannose oligosaccharides were generally attached in *S. cerevisiae* might be useful in furthering improvements in yeast-based GPCR analysis, for example, in engineering the enhancement of the glycosylation efficiency or to produce humanized glycoprotein in yeast cells (*29*). However, a multitude of reports on yeast-based GPCR analyses have demonstrated their availabilities (*4*, *5*, *7*, *30*); one, in particular, reported that the human

Table 2. Strains and plasmids.

Yeast strain or plasmid	Description
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
IM-50	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::AUR1-C ste2Δ::LEU2*
IMF-50	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::EGFP*
IMFD-50	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::EGFP his3Δ::P_{FIG1}-EGFP*
IMFD-70	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::EGFP his3Δ::P_{FIG1}-EGFP far1Δ*
pFIG1pt	P_{FIG1}*(300 bp)-*T_{FIG} in pCR4 Blunt-TOPO
pFIG1GF	P_{FIG1}*(300 bp)-EGFP-*T_{FIG1} in pFIG1pt
pMR-FIG1GF	P_{FIG1}*(300 bp)-EGFP-URA3-*T_{FIG1} in pFIG1GF [for generating *fig1Δ::EGFP* in IM-50]
pFIG450GF	P_{FIG1}*(450 bp)-EGFP-URA3-*T_{FIG1} in pUC119
pHIS3-FIG1GF	*HIS3(3′flanking region)-*P_{FIG1}*(450 bp)-EGFP-URA3-*T_{FIG1}*-HIS3(5′flanking region)* in pUC119 [for generating *his3Δ::*P_{FIG1}*-EGFP* in IMF-50]
pGK421	Yeast expression vector containing *PGK1* promoter, 2μ *ori* and *MET15* marker
pBlue-SSTR5-HA	*hSSTR5-HA* in pBluescript II KS (+)
pGK-SSTR5-HA	*hSSTR5-HA* in pGK421
pBlue-SSTR5-N13A	*hSSTR5(N13A)-HA* mutant in pBluescript II KS (+)
pBlue-SSTR5-N26A	*hSSTR5(N26A)-HA* mutant in pBluescript II KS (+)
pBlue-SSTR5-N13/26A	*hSSTR5(N13/26A)-HA* mutant in pBluescript II KS (+)
pGK-SSTR5-N13A	*hSSTR5(N13A)-HA* mutant in pGK421
pGK-SSTR5-N26A	*hSSTR5(N26A)-HA* mutant in pGK421
pGK-SSTR5-N13/26A	*hSSTR5(N13/26A)-HA* mutant in pGK421
pGK421-C-GFP	*EGFP* in pGK421
pSSTR5-GF	*hSSTR5-EGFP* in pGK421
pSSTR5-N13A-GF	*hSSTR5(N13A)-EGFP* in pGK421
pSSTR5-N26A-GF	*hSSTR5(N26A)-EGFP* in pGK421
pSSTR5-N13/26A-GF	*hSSTR5(N13/26A)-EGFP* in pGK421

Underlined strain and plasmids were used for assays.

adenosine A2a receptor could exert its function and localize in the plasma membrane without N-glycosylation in yeast (*31*). Even in the current study, somatostatin stimulation via human SSTR5 was coupled to the pheromone signalling pathway in yeast, exposing the apparent distinction of the signalling abilities between the wild-type receptor and its mutants.

Our work demonstrates the convenience of our yeast-based fluorescent signalling assay. In the case for somatostatin-specific signal transduction of rat SSTR3 in HEK293 cells, the inhibition of adenylyl cyclase activity was mediated by somatostatin-specific signalling via the α-subunits of the Gi family and the adenylyl cyclase activity-dependent cAMP concentrations were measured for quantification of the signalling (*19*). The determination of the cAMP production levels basically requires the activation of adenylyl cyclase with forskolin and a complicated multiple-step procedure, including cell washing, stopping cAMP accumulation, collecting intracellular cAMP with cell disruption, evaporation for cAMP enrichment, the use of a highly sensitive radiolabelled ligand and the generation of a standard curve. On the other hand, our yeast-based fluorescent signalling assay system could evaluate the somatostatin-specific hSSTR5 signalling with simple and instant manipulations merely by collection and direct analysis of non-destructive cells on the flow cytometer after incubation in a ligand-additive medium.

In conclusion, we showed the importance of two asparagine residues in the amino-terminus of human somatostatin receptor subtype-5 for somatostatin-specific signalling activation by using a yeast-based fluorescent reporter assay. This yeast-based system using a fluorescent reporter gene would be used by applying flow cytometric sorting to large-library screening, and will also be available for other human GPCRs and be beneficial for the simplification of experimental procedures in a GPCR signalling study.

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Conflict of interest

None declared.

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Control of signalling properties of human somatostatin receptor subtype-5 by additional signal sequences on its amino-terminus in yeast

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The yeast *Saccharomyces cerevisiae* is known as an available host for human G-protein-coupled receptor (GPCR) ligand screening. Although several types of yeast signal sequences (SS) attached with the GPCRs could improve their productivities and facilitate transportation of the GPCRs to the yeast plasma membrane, the effects of additional SS on ligand-specific signalling functions of GPCRs are not reported. Here, we demonstrated the controlling signalling properties by addition of SS using engineered yeast as a host. Prepro and pre regions of α-factor and amino-terminal sequence of Ste2 (Ste2N) were used as SS, and somatostatin (SST) receptor subtype-5 (SSTR5) was used as a model GPCR. We also constructed a yeast-based fluorescent assay system for monitoring the activation levels of SSTR5 signalling by a green fluorescent protein (*GFP*) reporter gene. The production levels and localization patterns of the SS-attached SSTR5 were more significantly improved than those of wild-type SSTR5. In addition, we successfully controlled the pharmacological efficacy and potency by introducing SS. Among four types of SSTR5 receptors, Ste2N–SSTR5 responded at the lowest ligand concentration. This finding will be informative for constructing optimal yeast-based ligand screening systems to discriminate the cells on the basis of signalling levels.

Keywords: fluorescent protein/G-protein-coupled receptor/signal sequence/somatostatin/yeast.

Abbreviations: G-protein, guanine nucleotide binding proteins, GPCR, G-protein-coupled receptor; SS, signal sequence, Ste2N, amino-terminal sequence of Ste2; SST, somatostatin, SSTR5, somatostatin receptor; SSTR5, somatostatin receptor subtype-5, GFP, green fluorescent protein; CFP, cyan fluorescent protein, RGS, regulator of G-protein signalling; *PGK5′*, *PGK1* promoter, *PGK3′*, *PGK1* terminator; *5′FIG1*, *FIG1* promoter, *5′GPA1*, *GPA1* promoter; *MCS*, multiple cloning site, *CEN/ARS*, centromere-autonomously replicating sequence; *EGFP*, enhanced green fluorescent protein, *ECFP*, enhanced cyan fluorescent protein; OD, optical density, MOPSO, 3-(N-morpholino)-2-hydroxypropanesulfonic acid; green-RFU, relative GFP fluorescence unit, cyan-RFU, relative CFP fluorescence unit; EC_{50}, half maximal effective concentration, Gi3tp, Gpa1-$G\alpha_{i3}$ transplant; prepro, prepro-α-factor, pre, pre-α-factor.

Somatostatin (SST) is a natural ligand known as a growth hormone release-inhibiting factor that regulates the human endocrine system by binding with somatostatin receptors (SSTRs) belonging to the G-protein-coupled receptor (GPCR) superfamily. Following natural ligand-binding, GPCRs can transduce external stimuli into internal signals via the intracellular heterotrimeric guanine nucleotide binding proteins (G-proteins) that comprise Gα-, Gβ- and Gγ-subunits (*1*). SSTRs are basically classified into five subtypes (SSTRx; x = 1–5), and they are widely expressed in several human organs with tissue-specific distribution patterns and significance for the therapeutic targets of several diseases (*2*). For example, SSTRs appear to predominantly regulate growth hormone secretion in acromegaly patients (*3*), and SSTR2 and SSTR5 in particular are expressed in almost every growth-hormone secreting tumor (*4–6*). Therefore, to support beneficial treatment of acromegaly patients, an easy-to-use system to screen selective drug candidates for each SSTR subtype is desired.

The eukaryotic unicellular yeast *Saccharomyces cerevisiae* is considered a powerful tool for human GPCR studies because it can simply examine signalling activation because of its uncompetitive and monopolistic G-protein signalling pathway (pheromone signalling pathway mediated by single yeast GPCR, Ste2 pheromone receptor) as compared to other higher mammalian cell lines (*7*, *8*). The yeast cells have successfully expressed many types of human GPCRs and have been adeptly used for various applications such as ligand screening and receptor mutagenesis using enzymatic and growth reporter genes (*9–11*). However, GPCRs seemingly include limitations for the arrangements of seven transmembrane structures and the signalling machineries in yeasts, and actually, some types of human GPCRs are still unreported to

transduce the signals responding to the agonists in yeast cells.

It has been reported that several types of signal sequences (SS) were attached to the amino-terminus to facilitate productivity and transportation of the receptors to the yeast plasma membrane. One of the most frequently-used leader sequences for the secretion of foreign proteins in yeast is the prepro region that comprises the amino-terminal 85 aa of the α-factor pheromone, which have been previously used to express heterologous GPCRs in yeast cells (*12*). The pre regions of several secretory proteins that are cleaved off by the signal peptidase are also often used for the protein secretions (*13*, *14*). In addition, the amino-terminal sequence of the yeast endogenous Ste2 receptor has been introduced to the amino-terminus of the heterologous receptors, such as human β_2-adrenergic receptor and human D_{2s} dopamine receptor (*15*, *16*). Although SS addition can improve GPCR productivity and transportation, the signalling functions of GPCR in yeast compared to natural GPCR without SS have not been reported.

In this study, we demonstrated controlling of signalling potency and efficacy in yeast by introduction of SS to the SSTR5 amino-terminus. For the applications to the practical primary screening of drug discovery, high sensitivity is needed even if lower ligand concentration (*i.e.* higher pharmacological potency). To monitor SSTR5 signalling activation levels, we used a yeast-based fluorescent signalling assay system that expresses green fluorescent protein (*GFP*) reporter gene in response to SST-specific SSTR5 activation. We first engineered the intracellular G-protein α-subunit to optimize the agonist-specific signalling function of human SSTR5 in yeast. We then evaluated the signalling activities of the natural form of SSTR5 receptor without SS and the engineered form of SSTR5 receptors with SS. In addition, we examined the SSTR5 receptor expression levels and localizations using a cyan fluorescent protein (CFP) tag protein fused to the SSTR5 carboxyl-terminus.

Materials and methods

Yeast strains and media

Yeast strains used in this study are listed in Table 1. *Saccharomyces cerevisiae* MI-170 is *gpa1*Δ *ste2*Δ *sst2*Δ *far1*Δ quadruple mutant strain, which was originated from the BY4741 starin. The *gpa1*Δ allele is deficient in the yeast Gα-subunit to introduce different types of Gα-subunits. The *ste2*Δ allele is deficient in the yeast single GPCR to express human receptor without competitive expression. The *sst2*Δ allele is deficient in the yeast principal negative regulator of G-protein signalling (RGS) to produce signalling hypersensitivity for the lower agonist concentration. The *far1*Δ allele is deficient in the yeast G1-cyclin-dependent kinase inhibitor to prevent the induction of G1 arrest in response to signalling.

As the initial host strain, the BY4741 *gpa1*Δ single mutant strain was used to generate MI-170 with homologous recombination and marker recycle method (*17*) using three types of DNA fragments: *5′STE2-URA3-hrSTE2-3′STE2*, *5′SST2-URA3-hrSST2-3′SST2* and *5′FAR1-URA3-hrFAR1-3′FAR1*. These fragments were amplified by PCR using suitable oligonucleotide primer pairs and DNA templates as listed in Tables 2 and 3. Sequential disruption of the *STE2*, *SST2*, and *FAR1* genes resulted in generation of the MI-100 (*gpa1*Δ *ste2*Δ), MI-150 (*gpa1*Δ *ste2*Δ *sst2*Δ), and MI-170 (*gpa1*Δ *ste2*Δ *sst2*Δ *far1*Δ) strains, respectively.

Yeast strains were cultivated in SD media containing 6.7 g/l yeast nitrogen base without amino acids (Becton, Dickinson and Company, Flanklin Lakes, NJ, USA), 20 g/l glucose and supplemented with appropriate amino acids and a nucleotide depending on the desired selectable marker (20 mg/l histidine, 60 mg/l leucine, 20 mg/l methionine and 20 mg/l uracil).

Plasmid constructions and yeast transformations

Plasmids constructed in this study are listed in Table 4. The DNA fragments were generated by PCR amplification from the respective template DNA using oligonucleotide primer pairs as listed in Tables 2 and 3, then inserted into cloning vectors at the corresponding restriction sites.

Eventually, the following 12 plasmids were used for the yeast transformations with the lithium acetate method (*18*). To investigate agonist-induced SSTR5 signalling activity, seven plasmids—pGK-SSTR5-HA, pGKalpha-SSTR5-HA, pGKal-SSTR5-HA, pGKst-SSTR5-HA, pMHG-FIG1, pSL-GPA1 and pSL-Gi3tp—were used to transform MI-170 (MI-170-*x*; $x = 1$–5) (Table 1). For estimation of receptor expression levels and observation of receptor localizations in yeast cells, five plasmids—pSSTR5-CF2, pSSTR5-CF2alpha, pSSTR5-CF2al, pSSTR5-CF2st and pGK421—were used to transform MI-170 (MI-170-*x*; $x = 6$–10) (Table 1).

Flow cytometric analysis

CFP and GFP fluorescence were, respectively, analysed on the BD FACSCanto II flow cytometer equipped with both a 405-nm violet laser and a 488-nm blue laser (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and the data were analysed using the BD FACSDiva software (v5.0; Becton, Dickinson and Company). The CFP fluorescence signal was collected through a 480/30 nm band-pass filter and the CFP-A mean of 10,000 cells was defined as 'cyan fluorescent intensity'; similarly, the GFP fluorescence signal was collected through a 530/30 nm band-pass filter and the GFP-A mean of 10,000 cells was defined as 'green fluorescent intensity'.

GPCR Signalling Assay

Yeast transformants (MI-170-*x*; $x = 1$–5) were grown in plain SD media (without buffering agent and pH control) at 30°C over night and were inoculated into 20 ml of the same SD media to give an initial optical density of 0.03 at 600 nm ($OD_{600} = 0.03$). The cells were then grown at 30°C on a rotary shaker at 150 rpm for up to 18 h and harvested. After washing, the cells were adjusted to give an $OD_{600} = 10$ with pH 7.1-adjusted SD media containing 200 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO). The yeast cell suspension (10 μl; to give an $OD_{600} = 1$) and the SST (14 aa peptide; Calbiochem, Darmstadt, Germany) (10 μl; to give an indicated final concentration, respectively) were added to the wells of 96-well cluster dishes containing fresh SD media (80 μl; adjusted to pH 7.1). The plates were incubated at 30°C and shaken at 150 rpm for 4 h. After incubation, the samples containing the yeast cells were diluted with 1 ml of sheath fluid, and GFP fluorescence was analysed by the flow cytometer. The relative GFP fluorescence units (green-RFU) were normalized by dividing the green fluorescent intensities of the yeast strains in the presence of SST by those of respective strains in the absence of SST. Assays were performed using three independent transformants. Half maximal effective concentrations (EC_{50}) values were determined using KaleidaGraph4.0 Fits to a dosersplgst model.

Determination of expression levels of SSTR5-CFP fusion proteins

Yeast transformants (MI-170-*x*; $x = 6$–10) were grown in plain SD media at 30°C overnight and the cells were inoculated into 20 ml of the same media to give an initial $OD_{600} = 0.03$. The cells were further cultivated at 30°C on a rotary shaker at 150 rpm for up to 18 h and then harvested. After washing, the cells were diluted with 1 ml of sheath fluid to give an $OD_{600} = 0.1$, and the CFP fluorescence was analysed with a flow cytometer. The relative CFP fluorescence units (cyan-RFU) were normalized by dividing the cyan fluorescent intensities of the yeast strains (MI-170-*x*; $x = 6$–9) by that of MI-170-10 control strain. Assays were performed using three independent transformants.

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Table 1. Yeast strains.

Strain	Relevant genotype	Reference or source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(*24*)
BY4741 *gpa1*Δ	BY4741 *gpa1*Δ::*kanMX4*	(*25*) purchased from Invitrogen, Carlsbad, CA, USA
MI-100	BY4741 *gpa1*Δ::*kanMX4 sst2*Δ	This study
MI-150	BY4741 *gpa1*Δ::*kanMX4 sst2*Δ *ste2*Δ	This study
MI-170	BY4741 *gpa1*Δ::*kanMX4 sst2*Δ *ste2*Δ *far1*Δ	This study
MI-170-1	MI-170 harbouring pMHG-FIG1, pSL-GPA1and pGKSSTR5-HA	This study
MI-170-2	MI-170 harbouring pMHG-IG1, pSL-Gi3tp and pGKSSTR5-HA	This study
MI-170-3	MI-170 harbouring pMHG-FIG1, pSL-Gi3tp and pGKakpha-SSTR5-HA	This study
MI-170-4	MI-170 harbouring pMHG-FIG1, pSL-Gi3tp and pGKal-SSTR5-HA	This study
MI-170-5	MI-170 harbouring pMHG-FIG1, pSL-Gi3tp and pGKst-SSTR5-HA	This study
MI-170-6	MI-170 harbouring pSSTR5-CF2	This study
MI-170-7	MI-170 harbouring pSSTR5-CF2alpha	This study
MI-170-8	MI-170 harbouring pSSTR5-CF2al	This study
MI-170-9	MI-170 harbouring pSSTR5-CF2st	This study
MI-170-10	MI-170 harbouring pGK421	This study

Fluorescence microscope observation of localization patterns of SSTR5-CFP fusion proteins

Yeast transformants (MI-170-*x*; $x = 6$–9) were grown in plain SD media at 30°C overnight, and the cells were inoculated into 20 ml of the same media to give an initial $OD_{600} = 0.03$. The cells were further cultivated at 30°C on a rotary shaker at 150 rpm for up to 18 h and then harvested. After washing, the cells were diluted with 1 ml of distilled water to give an $OD_{600} = 1$, and the CFP fluorescence images were analysed by a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Exposure time was 2 s for MI-170-6; and 1 s for MI-170-7, MI-170-8 and MI-170-9.

Results

Optimization of G-protein α-subunit for evaluating SST-specific SSTR5 signalling activity

To evaluate signalling activity of the SSTR5 responding to SST binding in yeast, we used a yeast-based fluorescent signalling assay system that induces expression of the *GFP* reporter gene by SST-dependent activation of GPCR signalling. To improve the functional coupling of the human SSTR5 to the yeast pheromone signalling pathway, the yeast–human chimeric Gα subunit, in which the carboxyl-terminal 5 aa residues of yeast endogenous Gα subunit (Gpa1) was substituted by that of human $G\alpha_{i3}$ (Gpa1-$G\alpha_{i3}$ transplant; Gi3tp), was produced as an alternative form of Gpa1 because of its effective signal transduction of the $G\alpha_{i3}$ familiar receptors (*19*). Then, the transduction efficiency coupled with the human SSTR5 was compared between Gpa1- and Gi3tp-expressing yeast cells (Fig. 1).

Figure 1A shows the dose–response curve for the pharmacological efficacy of ligand-specific SSTR5 signalling in yeast cells that express the endogenous Gα-subunit Gpa1 or the yeast–human chimeric Gα-subunit Gi3tp. In both cells, depending on the SST concentration, the appearance of green fluorescence was observed, clearly indicating that SSTR5 signalling is quantitatively measurable by the fluorescent signalling assay system using the *GFP* reporter gene. The maximum value for Green-RFU, which represented the maximum effect of the dose-response, was higher in the Gi3tp-expressing cells than in the Gpa1-expressing cells.

Figure 1B shows the pharmacological potency of ligand-specific SSTR5 signalling in Gpa1- or Gi3tp-expressing yeast cells. The higher EC_{50} value of the Gi3tp-expressing yeast cells than the Gpa1-expressing yeast cells suggests that the SST-induced interaction between the human SSTR5 and the yeast–human chimeric Gα subunit is more efficient for the signalling activation machinery on the intracellular peripheral yeast membrane (*19*). Therefore, for further experiments, Gi3tp-expressing yeast cells were used to investigate the effect of SS introduction to the SSTR5 amino-terminus on SST-dependent signalling activity in yeast.

Effect of yeast SS attachment at amino-terminus of human SSTR5 on SST-specific SSTR5 signalling activity in yeast

To investigate SST-specific signalling activity of human SSTR5 fused with SS at the amino-terminus in yeast, three types of yeast SS—prepro-α-factor (prepro), pre-α-factor (pre) and Ste2 amino-terminal 20 aa (Ste2N)—were selected because these particular SS could enhance the productivity and signalling abilities of human GPCRs in yeast cells (*12–16*). The three SS were genetically connected to the SSTR5 amino-terminus, and the SST-specific signalling activation levels of the SS-attached SSTR5 receptors were evaluated in the Gi3tp-expressing yeast cells using the *GFP* reporter gene.

Figure 2A shows the efficacies for SST-specific signalling activation mediated by SSTR5 and the three types of SS-SSTR5 receptors. SST-specific signalling activities were observed in all three SS-inserted SSTR5 receptors, although the maximal effects of the dose-responses (green-RFU) in the cells expressing the SS-attached SSTR5 receptors were lower than those of the cells expressing the natural SSTR5 receptor without SS addition. In the case of the Ste2N-SSTR5-expressing cells, the dose–response levels at lower SST concentrations were highest among all strains.

Figure 2B shows the pharmacological potencies of SST for the SSTR5 receptors expressed in yeast. The EC_{50} values of the cells expressing the pre-SSTR5 and Ste2N-SSTR5 were lower than those of the cells expressing the native form of the SSTR5 receptor,