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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 Asn26 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: *EGFP*, enhanced green fluorescent protein; FDA, Food and Drug Administration; 5-FOA, 5-fluoroorotic acid; GFP, green fluorescent protein; G-proteins, guanine nucleotide binding proteins; GPCR, G protein-coupled receptor; hSSTR5, human somatostatin receptor subtype-5; 2-ME, 2-mercaptoethanol; *PGK3′*, *PGK1* terminator; *PGK5′*, *PGK1* promoter; PMSF, phenylmethylsulphonyl fluoride; SD, synthetic dextrose; SSTR, somatostatin receptor; YPD, yeast extract–peptone–dextrose.

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

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 localisation 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, localisation 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 oligonucleotides used in this study were summarised in Table I. The strains and plasmids were summarised in Table II.

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

Table I. 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′-CCCCGGATCCTTTTTTTTTTTTTTTTTTGTTTG 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.

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

Table II. Strains and plasmids.

Yeast strain or plasmid	Description
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
IM-50	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::AUR1-C ste2Δ::LEU2*
IMF-50	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::EGFP*
IMFD-50	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::EGFP his3Δ::P_{FIG1}-EGFP*
IMFD-70	*MAT***a** *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.

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

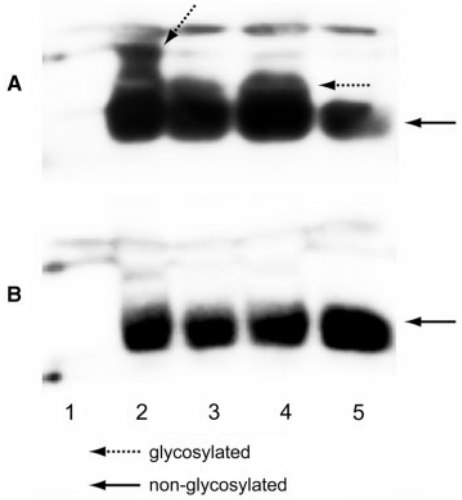

Fig. 1 Western blot analysis of hSSTR5s expressed in yeast. Membrane preparations of hSSTR5s in yeast transformants were analysed by SDS/PAGE (10% gel) and immunoblotted with the HA monoclonal antibody (**A**). The same samples were treated with endoglycosidase (Endo H_f) (**B**). Lane 1: mock, lane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

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.

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

To investigate the localisation 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

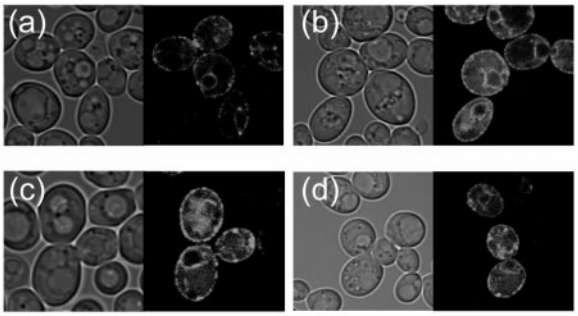

Fig. 2 Localisation of wild-type and mutated hSSTR5s. Wild-type and mutated hSSTR5s were fused with GFP at each carboxy terminus. Yeast cells expressing the GFP-fused hSSTR5s were grown in SD-selectable media for 18 h. The cell suspensions were observed with a confocal laser scanning microscope. (**A**)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

GFP-fused receptor expression plasmids. Green fluorescence was localised 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 localised on the surface membranes in the yeast cells. These results indicated that the mutation of Asn13 and Asn26 residues did not affect the localisation 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 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 (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

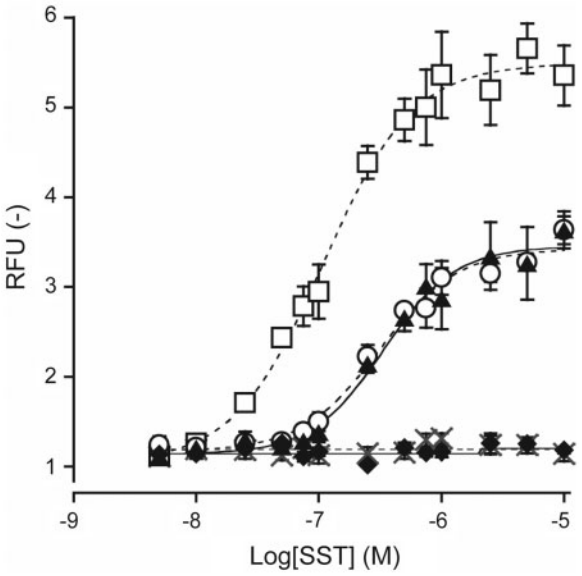

Fig. 3 Dose–response curves of signalling levels of wild-type and mutated hSSTR5s. All transformants were grown in SD selectable media for 18 h. The cell suspensions and each concentration of somatostatin were added into SDM71 media and incubated at 30°C for 4 h. The mean value of the green fluorescence 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.

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 adenosine A2a receptor could exert its function and localise 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 G_i 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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