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Monitoring of the tissue distribution of fibroblast growth factor containing a high mannose-type sugar chain produced in mutant yeast

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Most therapeutic glycoproteins have been produced in mammalian cell lines. However, the mammalian cell culture system has various disadvantages, *i.e.*, a high culture cost, difficulty in performing a large scale-up because of complicated handling requirements, and the risk of contamination by prion or other unknown pathogenic components through cultivation in the presence of bovine serum. There is thus a growing need for other host cells in which the recombinant glycoproteins can be produced. Recently, we successfully developed a mutant yeast strain engineered in a glycosylation system. The sugar chain produced in the mutant yeast is not immunogenic to the human immuno-surveillance system. In the present study, we selected fibroblast growth factor (FGF) as a model glycoprotein and assessed the bioactivity of FGF produced in yeast in terms of its proliferating activity and tissue distribution in mammalian cells and in the whole body. Structural changes in the sugar chains of FGFs derived from mutant yeast, as compared with those from mammalian cells, did not affect the proliferating activity remarkably. However, the tissue distribution in the mouse differed significantly; a high-mannose type sugar chain was the major determinant of the specific distribution of FGF to the kidney. The mechanism of this phenomenon is still unclear, but our observations suggest that recombinant glycoproteins derived from mutant yeasts producing high-mannose type sugar chains would be applicable for tissue-targeting therapy.
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Keywords: **fibroblast growth factor, *Saccharomyces cerevisiae*, glycoprotein, tissue distribution, tissue targeting therapy**

Abbreviations: **FGF, fibroblast growth factor; CHO, Chinese hamster ovary; 5-FOA, 5-fluoroorotic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HUVEC, human umbilical vein endothelial cell; DTT, dithiothreitol, BSA, bovine serum albumin.**

Introduction

At present, recombinant proteins produced in *Escherichia coli* are used in many fields for experimental, diagnostic, and therapeutic purposes. However, some *E. coli*-derived glycoproteins or non-glycosylated proteins are inadequate for *in vivo* use because of their rapid clearance from the blood, their potential for missing the target tissue, and their protease susceptibility. Thus, most therapeutic glycoproteins, such as erythropoietin, interferon and granulocyte colony stimulating factor, have been produced in mammalian cell lines, namely Chinese hamster ovary (CHO) cells and Baby hamster kidney cells [1–7]. However, there are some disadvantages to producing glycoproteins using mammalian cells. In many cases, mammalian cell culture requires fetal bovine serum as a supplement to the culture medium or serum-free synthetic-rich medium, and the costs are quite high. In addition, mammalian cell culture is complicated to handle and performing a large scale-up is difficult.

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Moreover, because of the recent problem of Bovine Spongiform Encephalopathy, the contamination of unknown serum factors must be excluded. There is thus an urgent need for alternative host cells that produce recombinant mammalian glycoproteins.

In the yeast *Saccharomyces cerevisiae*, N-linked oligosaccharide is classified into two categories, core oligosaccharide and outer chain, based on its biosynthesis and structure [8]. The dolichol-linked N-glycan synthetic pathway in the endoplasmic reticulum is identical in yeast and mammalian cells. In contrast, the N-linked sugar chain modification in the Golgi apparatus is generally different across species, organisms, cell strains and kinds of proteins. Unlike mammalian cells, the yeast *S. cerevisiae* extends the outer chain with a large α1,6-linked mannose backbone, which is further modified by the addition of α1,2- and α1,3-linked mannose side chains. This outer chain addition causes problems such as immunogenicity in humans and the disappearance of native glycoprotein activity [9,10]. Inactivation of the yeast-specific glycosylation system leads to the accumulation of an intermediate sugar chain common to humans. The introduction of mammalian glycosylation genes into this mutant yeast will make it possible to convert the intermediate sugar chains to various types of human sugar chains including chains that contain hybrid-type and complex-type oligosaccharides. In our previous study, we reported two yeast-specific glycosylation genes: the *OCH1* gene that encodes the initiation-specific α-1,6-mannosyltransferase and the *MNN4* gene that encodes a positive regulator of mannosylphosphorylation in yeast [11,12]. The mutant yeast, which is disrupted for these two genes, and the *MNN1* gene, which encodes yeast-specific α-1,3-mannosyltransferase, produced glycoproteins modified with intermediate sugar chains [12] that belong to the family of mammalian high-mannose type sugar chains.

The fibroblast growth factor (FGF) family consists of more than 20 members, and many studies have clarified their functions essential to regulating cellular differentiation, growth, development, wound healing and angiogenesis [reviewed in Ref. 13]. From our work on a complex carbohydrate remodeling project, we obtained some interesting results regarding the influence of the sugar chain on FGF functions. N-glycosylated FGF-6 produced by CHO cells potently induced DNA synthesis along with the proliferation of human umbilical vein endothelial cells (HUVEC), whereas in the absence of N-glycosylation, FGF-6 mitogenicity was substantially diminished [14]. In addition, FGF with multiple mucin-type O-glycans produced in CHO cells demonstrated mitogenic activity in vascular endothelial cells and had a longer lifetime in circulating blood [15]. Recently, Takamatsu *et al.* [16] discovered a linear correlation between the number of exposed galactose residues and the degree of distribution to the liver using branch-remodeled FGFs [16]. In the present study, we selected FGF as a model and examined the differences in biological functions between the products produced in CHO cells and yeast cells. The secFGF is a chimeric protein composed of mouse FGF-6 signal peptide and human FGF-1 core peptide [17]. It has two potential N-glycosylation sites (Asn35 and Asn149) on the polypeptide; however, the N-glycosylation site at Asn149 is glycosylated at a very low level [17].

Based on the information currently available, yeast *Saccharomyces cerevisiae* may be a useful host for heterologous expression of glycoproteins having exclusively oligomannosidic glycan chains but not of those having complex-type saccharide chains. In this report, we expressed secFGF in wild and mutant yeast cells that lacked yeast-specific mannosyltransferase genes. We designated sugar chain structures of secFGFs as one of three types, *i.e.*, mannan-type, high-mannose type, and complex-type. These three types consist of a high-mannose form having a large outer chain identical to that of the yeast *S. cerevisiae*, a high-mannose form not containing an outer chain, and a mammalian complex form, respectively. The secFGF produced by the mutant yeast contained mammalian high-mannose type sugar chains. In the tissue distribution analysis in the whole mouse body, we found that the secFGF produced by the mutant yeast was highly accumulated in the kidney. This accumulation was specific to the high-mannose type FGF. The affinity of the mannan-type FGF produced by wild-type yeast to the kidney was weaker than that of the complex-type FGF produced by CHO cells. Although the mechanism of this specific accumulation of the high-mannose type glycoprotein in the kidney remains unclear, these new findings should promote the use of mutant yeasts that produce high-mannose type sugar chains as host cells for the production of glycoproteins aimed at tissue-targeting therapy (as for example in the kidney).

Materials and methods

Strains and media

The genotypes of the *Saccharomyces cerevisiae* strains used or constructed in this work are summarized in Table 1. *S. cerevisiae* strains were grown in YPAD + 0.3 M KCl medium (1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose, 4 mg/l adenine, 0.3 M KCl) or SD + 0.3 M KCl medium (0.67% Bacto yeast nitrogen base, 2% dextrose, 0.3 M KCl) containing the nutritional supplements necessary to complement the strain auxotrophies or to allow the selection of transformants.

Disruption of the MNN1 gene

The three genes (*OCH1, MNN1* and *MNN4*) were disrupted by the method of Alani [18]. A cassette (HUH) in which the *Salmonella hisG* gene was linked to both ends of the *URA3* gene was digested by *Bgl*II and *Bam*HI from pNK51 [18], and was inserted into the *Bam*HI site of pSP73 (Promega Japan., Tokyo). The obtained plasmid was designated pSP73-HUH.

With the genomic DNA of W303-1A as a template, a 5′-untranslated region of the *MNN1* gene was amplified by PCR using the primers NOTPRO (GCGGCCGCGGATCCGAAGAAAACCTAATACATTGAAGT) and SPH5 (CCTTTGGTTTAATATAAATCTCCGGAGTGC). A 3′-untranslated region

Table 1. Strains used in this study

Strain	*Genotype*	*Source*
W303-1A	*MATa leu2-3,112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-100*	Kainuma *et al.* [20]
TIY1	W303-1A Δ*mnn1::HUH*	This study
TIY3	W303-1AΔ*mnn1::hisG*	This study
TIY9	W303-1A Δ*mnn1::hisG* Δ*mnn4::HUH*	This study
TIY11	W303-1A Δ*mnn1::hisG* Δ*mnn4::hisG*	This study
TIY17	W303-1A Δ*mnn1::hisG* Δ*mnn4::hisG* Δ*och1::HUH*	This study
TIY19	W303-1A Δ*mnn1::hisG* Δ*mnn4::hisG* Δ*och1::hisG*	This study
TIY42	W303-1A *secFGF::URA3*	This study
TIY43	W303-1A Δ*mnn1::hisG* Δ*mnn4::hisG* Δ*och1::hisGsecFGF::URA3*	This study
TIY44	W303-1A *secFGF::URA3 secFGF::LEU2*	This study
TIY45	W303-1A Δ*mnn1::hisG* Δ*mnn4::hisG* Δ*och1::hisG secFGF::URA3 secFGF::LEU2*	This study
TIY46	W303-1A *secFGF::URA3 secFGF::LEU2 secFGF::TRP1*	This study
TIY47	W303-1A Δ*mnn1::hisG* Δ*mnn4::hisG* Δ*och1::hisG secFGF::URA3 secFGF::LEU2 secFGF::TRP1*	This study
TIY48	W303-1A Δ*mnn1::hisG* Δ*mnn4::hisG* Δ*och1::hisG secFGF::LEU2*	This study
TIY53	W303-1A Δ*mnn1::hisG* Δ*mnn4::hisG* Δ*och1::hisG secFGF::LEU2 msdS::TRP1*	This study

of *MNN1* was also amplified by PCR using the primers SPH3 (TACATAACTCAATCAGCAGCAAATATGTC) and NOTEND (GCGGCCGCGTGTTCTGTTCGGGTAACGTTTAAACCAAT). These PCR products were treated with *Sph*I and *Not*I, and ligated into the *Sph*I site of pSP73-HUH. The resultant plasmid was linearized with *Not*I and was used for the transformation of the yeast W303-1A strain (*MATa leu2-3, 112 his3-11, 15 ade2-1 ura3-1 trp1-1 can1-100*). The transformation was done by the method of Ito *et al.* [19]. After the transformation, the cells were plated on SD-Ura (2% glucose, 1% casamino acids (Becton, Dickinson and Co., Sparks, MD), 0.67% yeast nitrogen base without amino acid (Becton, Dickinson and Co.), 40 mg/L adenine, 0.3 M KCl, 2% agar), and cultured at 30°C for 2 days. Incorporation of the *URA3* marker into the *MNN1* region in the chromosome of the transformant was confirmed by PCR using the above primers NOTPRO and NOTEND. This transformant was designated the TIY1 strain. To pop out the *URA3* marker, selection was carried out in a 5-FOA plate (0.67% yeast nitrogen base, 2% glucose, 40 mg/l adenine, 20 mg/l uracil, 2% agar) containing 0.1% 5-fluoroorotic acid (5-FOA), and a *URA3* gene-deficient strain was obtained. In the same manner as described above, the *mnn1* disrupted strain lacking the *URA3* gene was confirmed by PCR. This strain was designated TIY3.

Preparation of the double-disruptant strain (Δ*mnn1* Δ*mnn4*)

The nucleotide sequence of the *MNN4* gene was registered in the GenBank database under accession no. D83006. Using the genomic DNA of W303-1A as a template, a 5′-untranslated region of the *MNN4* gene was amplified by PCR using the primers SPEPRO (ATGGGCCCACTAGTATGCATCTCGCGTGGCATGG) and ECOEND (CCCCCGAATTCGTGTGAAGGAATAGTGACG). A 3′-untranslated region of *MNN4* was also amplified by PCR using the primers ECOPRO (CCCCCGAATTCAAGTCGGAGAACCTGACTG) and SPEEND (AGATGCATACTAGTGGGCCCATTGTGATTGGAAT). These PCR products were digested with *Eco*RI and *Spe*I, and ligated into the *Eco*RI site of pSP73-HUH. The resultant plasmid was linearized with *Spe*I and used for the transformation of TIY3. After the transformation, the cells were plated on SD-Ura medium and cultured at 30°C for 2 days. This transformant was designated the TIY9 strain. To pop out the *URA3* marker, selection was carried out as described above, and the *mnn1 mnn4* double-disrupted strain lacking the *URA3* gene was confirmed by PCR. This strain was designated TIY11.

Preparation of the triple-disruptant strain (Δ*och1* Δ*mnn1* Δ*mnn4*)

The nucleotide sequence of the *OCH1* gene was registered in the GenBank database under accession no. D11095. The disruption vector of *och1* was constructed as described by Nakayama *et al.* [11], except that the blunt-ended HUH cassette was ligated into blunt-ended *Hpa*I-*Aat*II sites. After the transformation, the cells were plated on SD-Ura medium and cultured at 30°C for 2 days. Incorporation of the *URA3* marker into the *OCH1* region in the chromosome of the transformant was confirmed by PCR. This transformant was designated the TIY17 strain. To pop out the *URA3* marker, selection was carried out as described above, and the *och1 mnn1 mnn4* triple-disrupted strain lacking the *URA3* gene was confirmed by PCR. This strain (Δ*och*1 :: *hisG*Δ*mnn*1 :: *hisG*Δ*mnn*4 :: *hisG*) was designated TI Y19.

Construction of the secFGF expression vector

pBSII-6N(mG+)1a [17], which contained an open reading frame of secFGF, was kindly provided by Dr. Imamura of Advanced Industrial Science and Technology, Tsukuba, Japan. The secFGF gene was removed from pBSII-6N(mG+)1a with *Sma*I

and *Nae*I, and was inserted into the *Hind*III site of pGEM2-alpha36, which harbors the yeast alpha-factor signal sequence. This construct was digested with *Eco*RI and subcloned into the *Eco*RI site of pUC119. This plasmid was designated FGF-pUC119.

To remove the C-terminal sequence (Glu-Ala-Glu-Ala) in the alpha-factor signal sequence, a truncated signal sequence was amplified by PCR with the M13 forward primer (CGCCAGGTTTTCCCAGTCACGAC) and alphaF-NaeI primer (ATGGGCCGGCTCTTTTATCCAAAGATAC). This product was cloned into the *Eco*RI site of pUC18, and the resultant plasmid was termed pAF02. pBSII-6N(mG+)1a was digested with *Nae*I and *Sma*I, and a part of the secFGF gene was inserted into the *Nae*I and *Sma*I sites of pAF02. The resultant plasmid was digested with *Eco*RI, and an open reading frame encoding the alpha-factor signal sequence without "Glu-Ala-Glu-Ala" fused to secFGF was cloned into the *Eco*RI site of the yeast expression vector YEp352GAP [20]. Finally, this plasmid was digested with *Aat*II and *Hpa*I to remove 2 μm DNA of YEp352GAP, after which the treatment with T4 DNA polymerase and the self-ligation were performed. This expression vector was designated pAFF3.

To integrate a few copies of the secFGF gene, we made other expression vectors that contained different auxotrophic markers. pAFF3 was digested with *Apa*LI and *Acc*I, and the fragment encoding the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, the alpha-factor signal sequence, secFGF, and the GAPDH terminator was transferred to the *Pvu*II sites of pYO324 and pYO325 that contained the *TRP1* and *LEU2* markers, respectively. The 2 μm DNA portions were removed from both plasmids with *Spe*I, and termed pAFF8 and pAFF9, respectively.

pAFF3, pAFF8 and pAFF9 were treated with *Eco*RV in order to be linearized, and were successively used for the transformation of the W303-1A and TIY19 strains. Transformation and confirmation of the gene integration were done as described above. The genotype of each strain is indicated in Table 1.

Co-expression of secFGF and α-1,2-mannosidase (MsdS protein)

The expression vector of *Aspergillus saitoi* α-1,2-mannosidase was described previously [21]. In order to integrate the *msdS* gene that encodes *A. saitoi* α-1,2-mannosidase, pGAMH1 plasmid was digested with *Eco*RI to remove the 2 μm DNA. This plasmid was designated pImsdS. pImsdS was linearized by *Xba*I at the position in the *TRP1* gene and used for the transformation of the TIY48 strain. The integration of the *msdS* gene into the transformant obtained from TIY48 was confirmed by PCR, and the resultant transformant was termed TIY53.

Purification of secFGF from cultural supernatant

The transformant was cultured in 3 liters of YPAD + KCl liquid medium at 30°C for 3 days. After centrifugation, the supernatant was recovered. Two milliliters of heparin-Sepharose (Amersham Biosciences Corp., Piscataway, NJ) was added to the supernatant and stirred gently overnight. Heparin-Sepharose was packed into an HR5/2 column (Amersham Biosciences Corp.) and was washed with HS buffer (10 mM phosphate buffer containing 2.7 mM KCl and 0.137 M NaCl, 0.01% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), pH 7.4) using a SMART system (Amersham Biosciences Corp.). secFGF was eluted by increasing the NaCl concentration up to 2.5 M in the HS buffer. The secFGF fraction was again subjected to heparin-Sepharose chromatography. The eluted secFGF was applied to a μRPC C2/C18 PC 3.2/3 column (Amersham Biosciences Corp.) pre-equilibrated with solvent A (0.1% trifluoroacetic acid) to remove NaCl. After washing the column with solvent A, secFGF was eluted with a linear increase of solvent B (60% acetonitrile 0.1% trifluoroacetic acid) up to 100%. The eluted secFGF fraction was evaporated and used for sugar chain analysis and tissue distribution analysis.

SDS-PAGE and western blotting

SDS-polyacrylamide gel electrophoresis and western blotting were done as described previously [21,22].

Growth stimulating activity assay

FGF-1 produced in *E.coli* was purchased from R&D Systems (Minneapolis, MN). The glycosylated (mainly biantennary sugar chain) and the non-glycosylated secFGFs derived from CHO cells were kindly donated by Dr. Imamura (Advanced Industrial Science and Technology, Tsukuba, Japan).

The branch structures of sugar chains of secFGF produced in CHO cells were as follows: biantennary: 66.1%; triantennary (GlcNAcβ1,4-branched): 2.6%; triantennary (GlcNAcβ1,6-branched): 17.4%; and tetraantennary: 3.3% [16]. Non-glycosylated secFGF was prepared by PNGase F digestion [16]. The growth stimulating assay using HUVEC were performed as described below. HUVEC (HUE127-1) was also kindly provided by Dr. Imamura (AIST, Tsukuba, Japan). HUVEC was cultured in M199 medium supplemented with 15% FBS, 5 μg/ml heparin and 5 ng/ml recombinant FGF-1 in a 5% CO_2 humidified atmosphere at 37°C. 2×10^3 cells were seeded in 96-well plates and incubated for 24 h. The cells were washed twice with heparin and recombinant FGF-1 free medium and further incubated for 24 h. Various concentrations of the secFGFs with or without 10 μg/ml heparin were then added to each plate, and the cells were incubated for an additional 72 h. Heparin was added to stabilize secFGF, as well as to support FGF-FGF receptor binding for assistant factor. The cell numbers were estimated using a TetraColor One Kit (Seikagaku Corp., Tokyo, Japan). In brief, 10 μl of TetraColor One solution was added to each plate, and the plates were incubated for 2 h at 37°C. Absorbance at 450 and 655 nm was measured and plotted the ratio of absorbance at 450 to 655 nm.

Sugar chain analysis

N-linked oligosaccharides were released from FGF by hydrazinolysis using a Hydraclub and a hydrazinolysis reagent C kit (Honen Corporation, Tokyo, Japan). The recovered oligosaccharides were labeled with 2-aminopyridine using a commercially available reagent kit (TAKARA, Shiga, Japan). After pyridylamination, the samples were purified by gel filtration with a TOYOPEARL HW-40 column (Tosoh Corp., Tokyo, Japan).

Separation of oligosaccharides was carried out by HPLC using a Waters LC Module I plus a chromatograph system equipped with a UV spectromonitor or fluorescence spectromonitor FS-8020. Size-fractionation HPLC was performed with Asahipak NH_2P-50 (4.6×250 mm) at a flow rate of 1.0 ml/min. The column was equilibrated with 100% of solvent A (200 mM acetic acid/triethylamine (pH 7.3): acetonitrile = 3:7). After the sample injection, the proportion of solvent B (200 mM acetic acid/triethylamine (pH 7.3): acetonitrile = 2:8) was increased linearly up to 100% in 50 min. The proportion of solvent B remained at 100% from 50 to 70 min.

Tissue distribution analysis

secFGFs were iodinated using IODO-BEADS Iodination Reagent (PIERCE, Rockford, IL). One of the IODO-BEADS was added to a solution of 200 μCi of $Na^{125}I$ in 180 μl of 0.1 M sodium-phosphate buffer, pH 6.5, and allowed to react for 5 min at 25°C. Then, 20 μg of secFGF in 20 μl of PBS was added to the reaction mixture. The reaction was stopped by removing the IODO-BEADS from the reaction mixture. Radioiodinated secFGFs were purified by the following method. In brief, labeled secFGFs were applied to a heparin-Sepharose CL-6B column equilibrated with 20 mM HEPES containing 0.1 mM dithiothreitol (DTT), pH 7.3, and washed once with the same buffer. The column was then washed with 20 mM HEPES buffer containing 0.1 mM DTT and 0.5 M NaCl, pH 7.3, and the labeled secFGFs were eluted with 20 mM HEPES buffer containing 0.1 mM DTT and 1.5 M NaCl, pH 7.3. The labeled secFGF samples were diluted with PBS containing BSA (final concentration: 0.25% ⟨w/⟩) and injected into male ddY mice (20–25 g body weight) via a lateral tail vein. At selected times, the organs of interest were removed after exsanguination from the heart under ether anesthesia. Blood and tissue samples were weighed, and their radioactivities were measured with a well-type scintillation counter (ARC300; Aloka, Tokyo, Japan). Tissue distribution was expressed as a percentage of the dose.

Results

Multiple gene disruption with the Salmonella hisG gene

To disrupt a yeast-specific glycosyltransferase gene, we had initially used a conventional method. By the conventional method, however, we had to use three markers to construct a triple disruptant (*och1 mnn1 mnn4*), since each marker gene would only disrupt a single gene. In addition, multiple insertions of the genes were required in order to make a human-type sugar chain in *S. cerevisiae*. We therefore switched to a disruption method using a *Salmonella hisG* gene developed by Alani *et al*. [18]. The outline of this method is shown in Figure 1. A *Salmonella hisG-Saccharomyces URA3-Salmonella hisG* cassette was inserted into the target gene, and this construct was used to transform the ura3$^-$ strain. The transformant would show an Ura$^+$ phenotype. After confirmation of the gene disruption by PCR (data not shown), the transformant was inoculated on the plate containing 5'-fluoroorotic acid (5-FOA). Because 5-FOA is toxic for the Ura3$^+$ strain, only Ura3$^-$ cells produced due to the lack of the *URA3* gene would survive. Alani's method easily eliminates the Ura$^+$ marker by the high-frequency recombination between the flanking direct repeats [18]. In this manner, we constructed a triple disruptant mutant, TIY19 (*och1::hisG mnn1::hisG mnn4::hisG*) (Table 1), which was confirmed by sequencing the corresponding region, and used it for the production of secFGF.

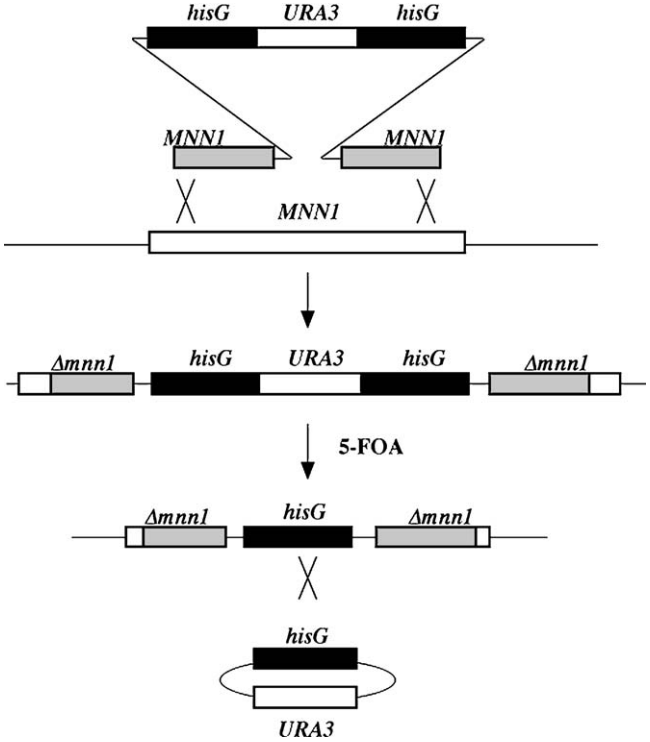


Figure 1. Schematic representation of disruption of the target gene in *Saccharomyces cerevisiae* using a *hisG-URA3-hisG* cassette.

Production of the secFGF chimeric protein

For the secretion of secFGF protein in *S. cerevisiae* mutant cells, the secFGF construct was fused after the alpha-factor prepro sequence. It is known that the alpha-factor prepro sequence is removed by yeast Kex2 protease via post-Golgi modification. The kex2 protease recognizes the sequence of

"Lys-Arg-Glu-Ala-Glu-Ala" in the alpha-factor prepro sequence and cuts between Arg and Glu. Although the resultant tetrapeptide (Glu-Ala-Glu-Ala; EAEA) in the nascent protein is removed by the protease encoded by the *STE13* gene, the efficiency of this process is not sufficient due to the low activity of Ste13 protease when the products are overproduced. Therefore, we constructed an secFGF expression vector lacking the above EAEA sequence.

The construct was inserted downstream of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter in YEp352GAP [20]. As it is more stable to integrate the gene in the chromosome than to harbor the gene as a plasmid, an integration vector was constructed by removing the 2 μm DNA fragment from YEp352GAP-secFGF. We also constructed the other integration vectors containing the *LEU2* gene or the *TRP1* gene in a similar way. Each vector was used for the successive transformation of yeast, and the cells having two or three copies of the secFGF gene were developed. Integration of the secFGF genes in each locus was confirmed by genome PCR (data not shown).

The amounts of secFGF produced in each strain were compared by western blotting (Figure 2). In both the wild-type and disruptant strains, the signal intensity of FGF was increased depending on the number of copies of secFGF genes. In the wild-type strains (TIY42, 44 and 46), the bands were smeared, and the apparent molecular mass was larger than the expected one calculated from the protein sequence. Because the signal was shifted to a lower molecular mass by the treatment with endoH (data not shown), it is likely that the higher and heterogeneous molecular mass was caused by the elongation of the outer chain of N-glycan. On the other hand, one major signal and two minor signals were observed in the triple disruptant strains (TIY43, 45 and 47). The major signal was shifted to the position corresponding to the non-glycosylated one by digestion with endoH (data not shown). Therefore, it is likely that the major signal was the secFGF protein containing the high-mannose type sugar chain.

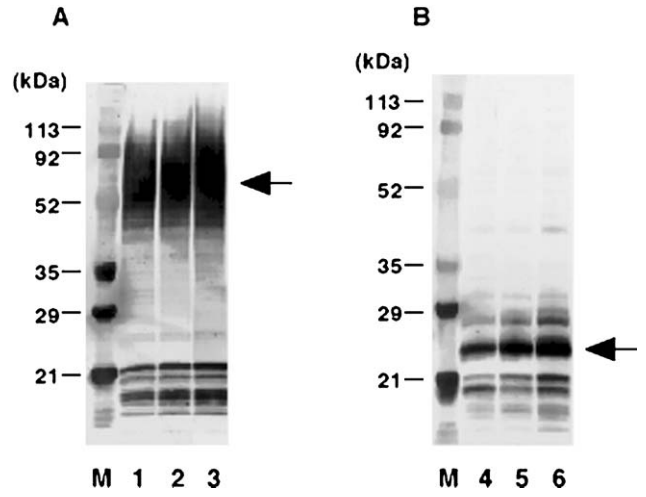


Figure 2. Western blot analysis of the recombinant secFGF produced in wild-type (A) and mutant (B) yeast. Fifteen microliters of culture supernatant were applied to each lane. M: molecular marker. Lane 1: TIY42; lane 2: TIY44; lane 3: TIY46; lane 4: TIY43; lane 5: TIY45; lane 6: TIY47. Arrows indicate the position of secFGF.

The properties of secFGF produced by the disruptant yeast

To purify secFGF, 3 liter cultures of the TIY46 and TIY47 strains were used. After 3 days of culture in 3 liter of complete medium, heparin-Sepharose was added to the culture supernatant, and secFGF was adsorbed to the heparin-Sepharose by overnight shaking at 4°C. The heparin-Sepharose was packed into a column and eluted with buffer containing 2 M NaCl. A single peak was detected with both the TIY46 and TIY47 strains, and their purities were confirmed by SDS-PAGE (data not shown).

The N-terminal amino acid sequence was checked by the Edman degradation of secFGF purified from the TIY47 supernatant. The amino acid sequence was assigned as Ala-Gly-Ala-Arg-Ala-Xaa-Gly-Thr-Leu-Leu-Asp-Ala (Xaa means unknown), which was identical to the N-terminal sequence of secFGF. However, a sixth amino acid residue (Asn) was not identified. Because the Asn-X-Thr is a potential N-glycosylation site, the results indicated that the Xaa should be Asn, which is modified with the *N*-linked sugar chain, to be consistent with the results of western blotting.

We also determined the structure of high-mannose type oligosaccharides attached to the secFGF that was produced in W303-1A (wild-type) and TIY47 cells (Figure 3). As expected, many peaks were observed at the elution positions larger than $Man_9GlcNAc_2$-PA in the wild-type strain (Figure 3A), while the sugar chains of secFGF from TIY47 were separated into four major peaks (Figure 3B). After comparing these findings with those for the authentic samples that were prepared from *S. cerevisiae* oligosaccharide mutants, we identified the first and second peaks eluted at 28 to 33 min as neutral sugars ($Man_8GlcNAc_2$-PA and $Man_9GlcNAc_2$-PA), and the third and fourth peaks (eluted at 46 and 51 min) as yeast-specific phosphorylated sugars (Man-P-$Man_8GlcNAc_2$-PA). Based on the peak area, the relative ratio of the neutral and the acidic sugars was 7 to 3. The remaining three or more minor peaks could not be defined.

Finally, we compared the effects of the two secFGFs on DNA synthesis in HUVEC (Figure 4). The recombinant FGF-1 produced in *E. coli* and the recombinant non-glycosylated secFGF produced in CHO cells showed almost the same activity for *in vitro* growth of HUVEC. Although the two secFGFs produced in yeast potently induced proliferation of HUVEC, the effect of *in vitro* growth stimulation was less than that of the non-glycosylated secFGF. At the concentration of 3 ng/ml, the growth-stimulating activities of the secFGF with high-mannose type (mutant-type) and with mannan-type (wild-type) oligosaccharides decreased about 75 and 60%, respectively, compared with the non-glycosylated form of FGF.

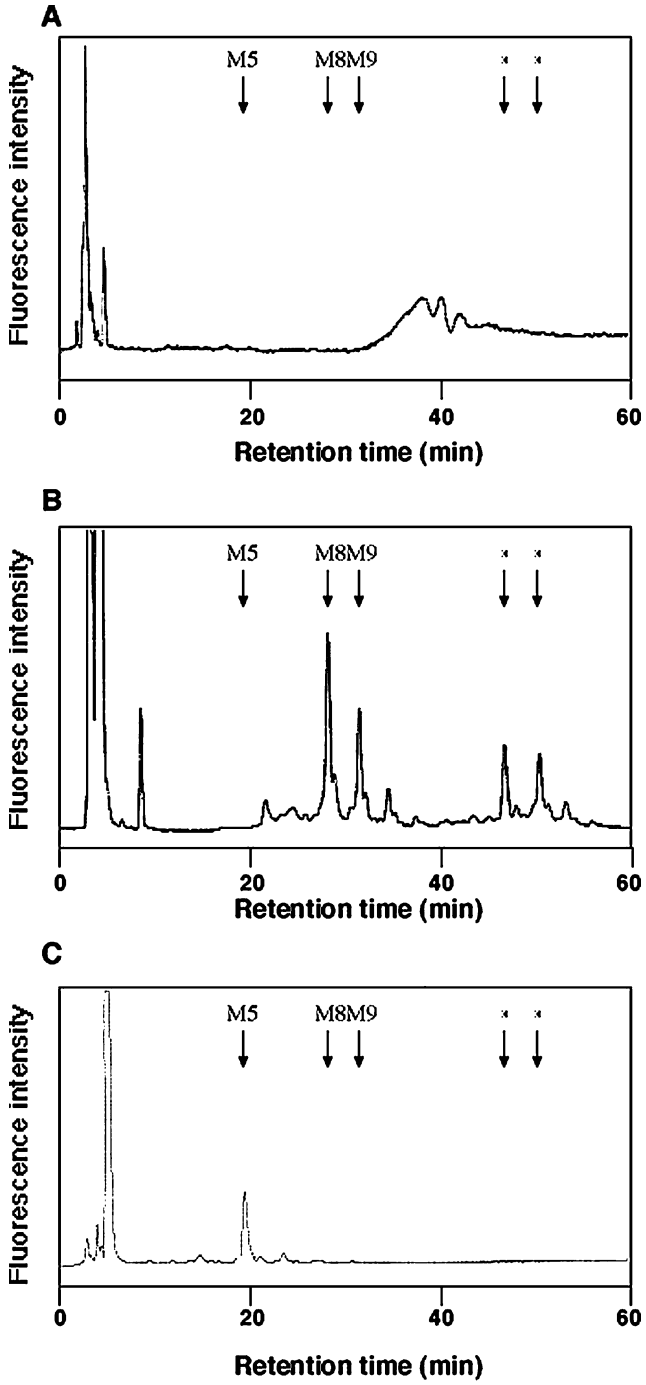


Figure 3. HPLC profiles of the PA-labeled sugar chain of the secFGFs produced in yeast strains TIY42 (A), TIY47 (B) and TIY53 (C). Arrows indicate the position of the authentic PA-sugar chain. M5: $Man_5GlcNAc_2$-PA; M8: $Man_8GlcNAc_2$-PA; M9: $Man_9GlcNAc_2$-PA; *: Man-P-$Man_8GlcNAc_2$-PA.

Production of secFGF with $Man_5GlcNAc_2$-type oligosaccharide

In order to analyze the function of the oligosaccharide portion of secFGF, another secFGF containing $Man_5GlcNAc_2$-type oligosaccharide was produced using a method reported previously [21]. The secFGF gene and the α-1,2-mannosidase gene (*msdS*) from *Aspergillus saitoi* were co-introduced into the TIY19 cells, and the recombinant clone was termed TIY53. The production and purification of secFGF from TIY53 cells were the same as described above. The oligosaccharide structure of secFGF from TIY53 was also determined. The HPLC pattern (Figure 3C) indicated that the main structure was $Man_5GlcNAc_2$-PA, and that no acidic oligosaccharide portion existed.

Influence of sugar chain structure on the tissue distribution of secFGF in the mouse

To examine the function of glycan structures in FGF, we analyzed the tissue distribution of the secFGFs that were derived from CHO and yeast cells. We used glycosylated and non-glycosylated (*in vitro* PNGase-digested) forms of secFGF produced using CHO cells and three types of those produced using mutant yeast cells (a wild-type, Δ*och1* Δ*mnn1* Δ*mnn4* triple mutant, and Δ*och1* Δ*mnn1* Δ*mnn4* triple mutant harboring the α-1,2-mannosidase expression vector). From the structural analysis, the sugar chains of secFGF obtained from CHO, wild-type yeast, and two mutant-type yeasts were confirmed as an almost biantennary type, a mannan-type, and a high-mannose type, respectively (data not shown). Thus, the secFGFs were designated Bi-FGF, Wild-FGF, and M8- and/or M5-FGF, respectively. The secFGFs have eight tyrosine residues in their polypeptide sequences [17], and some of these residues can be labeled with ^{125}I. We examined the tissue distribution of these secFGF glycoforms in the mouse by using the radiolabeled proteins (Figure 5). The blood clearance of the secFGFs was very rapid, and the radioactivity in the blood decreased to between 2–20% of the dose at 5 min after intravenous injection with any of the secFGF glycoforms (Figure 5A). It is well known that the secFGFs disappear more rapidly from the blood circulation than another glycoprotein, IFN-γs [16]. Even so, Bi-FGF was cleared a little more slowly than either the non-glycosylated form or Wild-FGF. Blood clearances of the M8- and M5-FGF were very rapid. For M5- and M8-FGF in particular, less than about 2% of the dose remained at 5 min after the injection. The blood clearance of Wild-FGF was intermediate between those of Bi-FGF and the M5- and M8-FGFs. The radioactivity in the stomach increased after 15 min, suggesting that the released $^{125}I^-$ or small molecules containing ^{125}I degraded from ^{125}I-secFGFs accumulated in the stomach (Figure 5D). It is likely that the labeled compounds of yeast-derived secFGFs are more stable than those of CHO-derived secFGFs. Except in the stomach, the radioactivity was mainly distributed to the liver and kidney. A substantial accumulation of secFGF was also seen in the spleen, which was especially significant in the case of CHO-derived secFGFs (data not shown).

Five minutes after injection, most of the radioactivity was found in the liver (Figure 5C). Based on the reciprocal tendency between the radioactivity in the liver and that in the blood (Figure 5A and C), we considered that the clearance from the

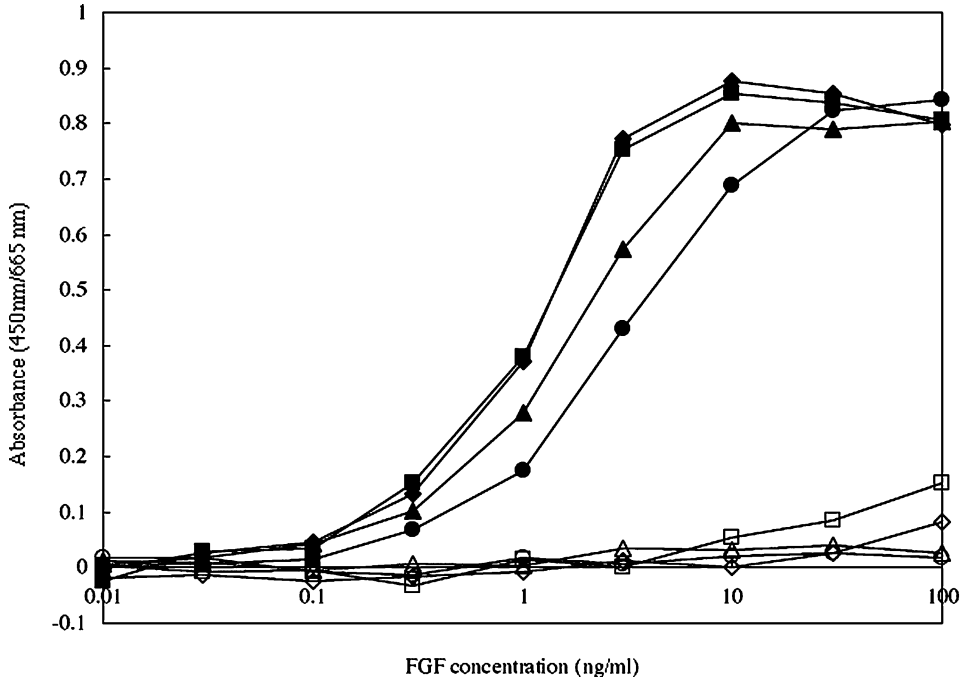


Figure 4. HUVEC proliferation activities of secFGFs. HUVEC were cultivated with various secFGFs in the presence or absence of heparin. Open square: non-glycosylated secFGF produced in CHO cells in the absence of heparin. Open diamond: FGF-1 produced in *E. coli* in the absence of heparin. Open circle: secFGF with mannan-type N-glycan produced in the absence of heparin. Open triangle: secFGF with high mannose-type N-glycan (M8) produced in the absence of heparin. Closed square: non-glycosylated secFGF produced in CHO cells in the presence of heparin. Closed diamond: FGF-1 produced in *E. coli* in the presence of heparin. Closed circle: secFGF with mannan-type N-glycan produced in the presence of heparin. Closed triangle: secFGF with high mannose-type N-glycan (M8) produced in the presence of heparin. Results shown are representative of three separate replications. Each point represents the mean of data tested in duplicate.

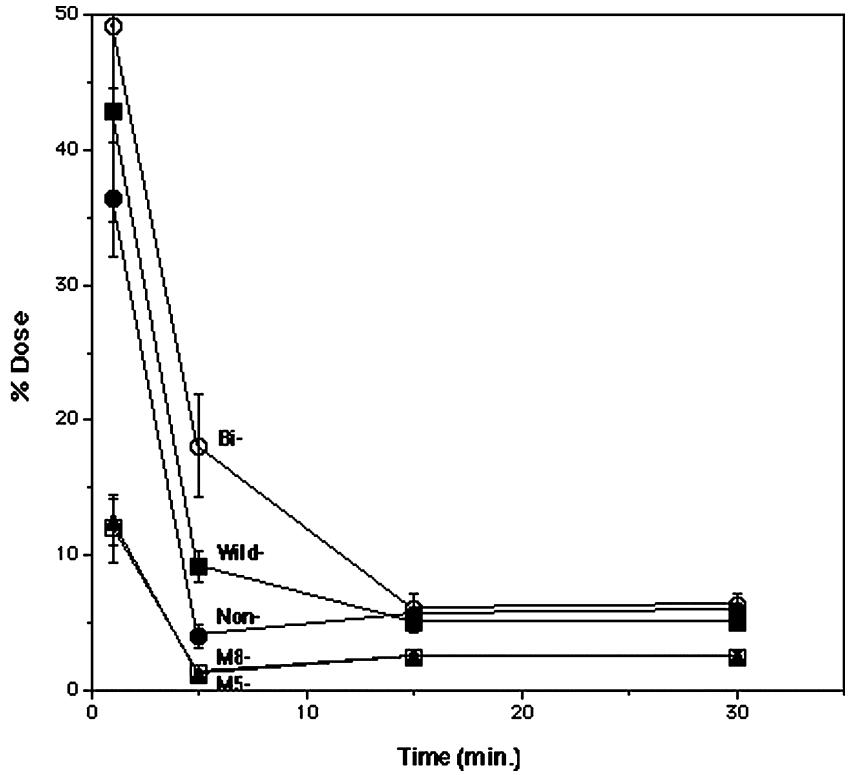
blood seemed to be largely affected by the hepatic uptake. We assume that the recognition of the exposed mannose residues on secFGFs by hepatic mannose receptor was responsible for this result. As the CHO-derived secFGFs (i.e., the complex-type and the non-glycosylated form) are also accumulated in the liver, it is likely that another recognition mechanism for secFGFs exists in the liver. To examine the effect of the number of mannoses in the high mannose-type sugar chains, we also investigated the tissue distribution of M5- and M8-FGFs. The blood clearance curve and the accumulation patterns in the liver and kidney of M5-FGF were close to those of M8-FGF.

When we examined the distribution of secFGF to eight tissues—the brain, bone, lung, liver, kidney, spleen, stomach, and intestine—at 5 min after intravenous injection, the amount of secFGF was greatest in the liver, followed by the kidney. Because the blood contamination in the kidney was negligible, the distribution was considered to be due to the uptake by the kidney itself. It is notable that the concentrations of M8- and M5-FGFs in the kidney were much higher than those in the blood (Figure 5A and B). In the case of M8- and M5-FGFs, the concentrations in the kidney (radioactivity per mg tissue) were more than 10 times higher than the concentration in the blood (radioactivity per ml blood) at 5 min after injection, while in other forms, the ratios were not more than 3 times higher. In particular, the concentration in the kidney of Wild-FGF was only half of the concentration in the blood. Thus, it is likely that the M8- and M5-FGFs have a specific affinity for the kidney, although secFGF as a whole glycoprotein also seems to have a low level of affinity for the kidney.

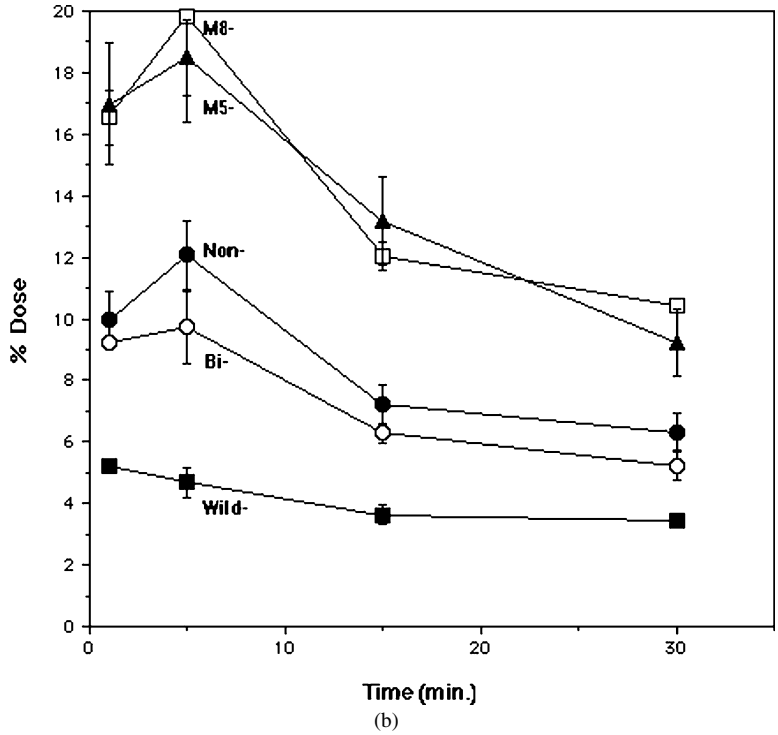
The distribution of secFGFs to mouse organs other than the liver and kidney was low (less than 5% of the administered dose), and the difference among various secFGF forms was relatively small, except that the levels of Bi-FGF and non-glycosylated secFGF in the spleen were relatively high (data not shown).

Discussion

In this paper, we reported the production of recombinant secFGFs in yeast, and examined the tissue distribution of secFGF containing a high-mannose type sugar chain. When we introduced the secFGF gene that was fused to the native alpha-factor signal sequence into wild-type yeast, an extra sequence consisting of four amino acid residues (EAEA) was added to the *N*-terminus of the produced FGF6/1 (data not shown). We therefore constructed a new fusion gene that contained both the alpha factor signal sequence lacking EAEA and secFGF. The *N*-terminal amino acid sequence analysis of secFGF showed

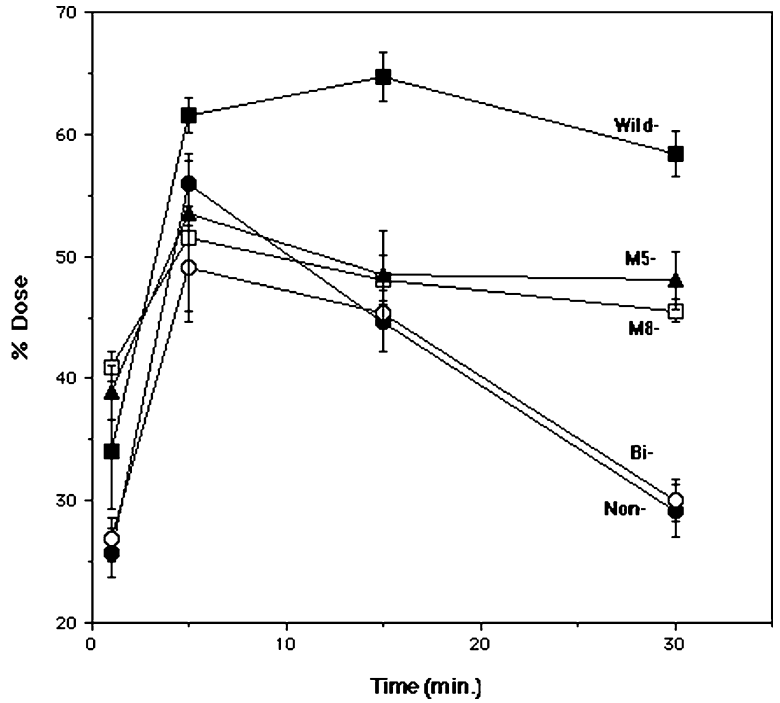


(a)

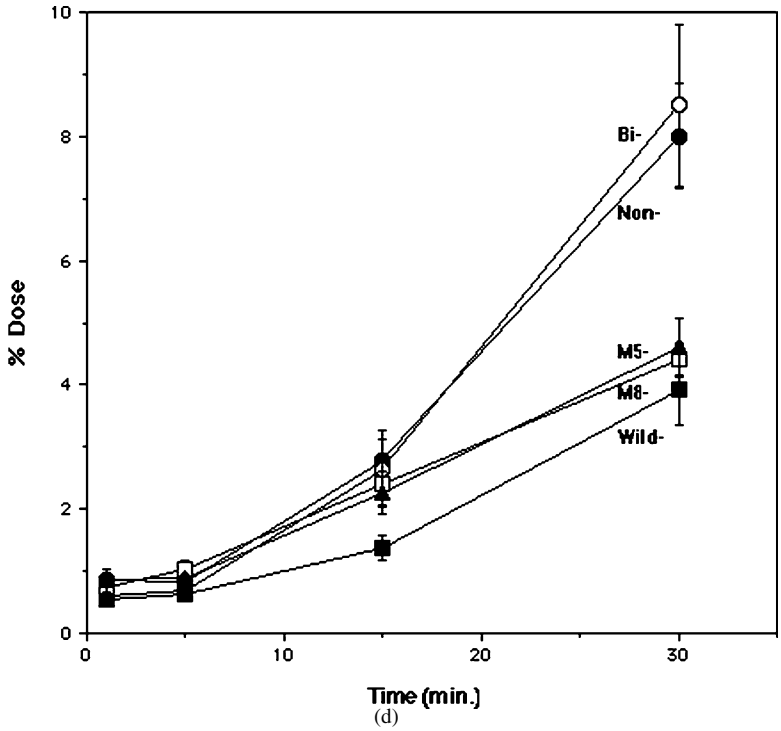


(b)

Figure 5. Time course of radioactivity after intravenous administration of ^{125}I-labeled secFGFs in mice. (A) Blood, (B) Kidney, (C) Liver, (D) Stomach. losed circle (Non-): non-glycosylated secFGF produced in CHO cells. Open circle (Bi-): secFGF produced in CHO cells (Bi-FGF). Closed square (Wild-): secFGF with mannan-type *N*-glycan produced in the TIY46 strain (Wild-FGF). Open square (M8-): secFGF with high-mannose type N-glycan (M8) produced in the TIY47 strain (M8-FGF). Closed triangle (M5-): secFGF with high-mannose type N-glycan (M5) produced in the TIY53 strain (M5-FGF). Results are expressed as the mean ± S.D. of five mice. (*Continued on next page.*)



(c)



(d)

Figure 5. (*Continued.*)

that the Kex2 protein efficiently recognized the Lys-Arg dipeptide sequence and digested secFGF after the Arg residue. This result indicated that the removal of the EAEA sequence was effective to make a protein with a homogeneous *N*-terminal sequence in yeast cells.

The secFGF has two potential N-glycosylation sites (Asn35 and Asn149), and a major portion of the secFGF expressed in yeast was of the one-glycosylated form (Figure 2). The analysis of the *N*-terminal amino acid sequence of secFGF also indicated N-glycosylation at Asn35. The efficiency of the glycosylation

of Asn149 in the C-terminal region of secFGF seemed to be low. Yoneda *et al*. showed that when Asn35 of FGF was substituted for Gln, the mutated FGF with only one potential N-glycosylation site (Asn149) was secreted from Ba/F3 cells with little glycosylation [17]. Studies on other proteins have similarly reported that the Asn-X-Ser/Thr sequence near the C-terminus was likely to be less glycosylated [23–25]. It has been reported that the minimum distance of an acceptor site from the lumenal end of a transmembrane segment was 12–14 amino acids in length, suggesting that the active site of the oligosaccharyltransferase was positioned 30–40 Å above the ER membrane surface [26]. In our case, as the Asn 149 was the 27th amino acid from the C-terminus of secFGF, the reduced glycosylation of Asn149 was likely due to the physical constraints of nascent secFGF on glycosyl transfer.

Comparing glycosylated and non-glycosylated secFGFs, the growth-stimulating activities of M8-FGF and Wild-FGF were a little weaker than those of the *E. coli*-derived non-glycosylated FGF-1 and the PNGase-digested secFGF (Figure 4). A decrease of growth-stimulating activity was also observed for Bi-FGF compared with the *E. coli*-derived non-glycosylated form (data not shown). Since natural FGF-1 has no carbohydrate moiety, we considered that the N-glycan attachment to secFGFs influenced their affinity for receptor binding and reduced their proliferating activity. That is, the bulkiness of the mannan-type sugar chain relative to the high-mannose type sugar chain may have resulted in the lower proliferating activity of the Wild-FGF relative to M8-FGF.

Although TIY49 was disrupted with the *MNN4* gene, it produced secFGFs containing both neutral and acidic sugar chains. In a previous paper, we reported that YS132-8B, which was disrupted with the *OCH1, MNN1* and *MNN4* genes, produced only the $Man_8GlcNAc_2$-type sugar chain, and the acidic-type sugar chain was not detected [21]. Because TIY49 was derived from the W303-1A strain and YS132-8B was derived from the YPH500 strain, the difference in the composition of the sugar chains between TIY49 and YS132-8B was likely to be caused by the difference in the parental strains. On the other hand, TIY53 produced secFGF having only $Man_5GlcNAc_2$-type sugar chains, and other larger and acidic sugar chains were not observed. This was quite different from the sugar chain patterns of CPY and the mannoproteins from YS132-8B/pGAMH1 [21]. Sugar chain analysis of mannoproteins from TIY53 revealed heterogeneous sugar chains ($Man_{5-8}GlcNAc_2$; data not shown). Because the recombinant secFGF from CHO cells contained mainly sialylated biantennary sugar chains [16], the efficiency with which the expressed α-1,2-mannosidase trimmed the sugar chains was likely to be affected by the accessibility of the mannosidase to the sugar chains in TIY53.

We produced three types of secFGF (Wild-FGF, M8-FGF and M5-FGF) using wild-type and mutant yeast cells. To study the effects of modified sugar chains on secFGFs, we assessed the bioactivities, including the proliferating activity and tissue distribution, then compared them with those of CHO-derived secFGF and deglycosylated secFGF. Recently, Takamatsu *et al*. [16] discovered a linear, but not exponential, enhancement of the distribution of FGF to the liver with an increase in the number of exposed galactose residues. This phenomenon was considered to be mediated by the hepatic asialoglycoprotein receptor, but was different from the ordinary "glycoside cluster effect" [27]. Three types of secFGF produced in yeast have exposed mannose residues. It is well known that the elimination of glycoproteins from the blood by the liver is dependent on galactose receptors (or asialoglycoprotein receptors) and mannose receptors [28,29]. Thus, we postulated that the Wild-, M8- and M5-FGFs would be highly accumulated in the liver. The results were consistent with our expectations. Wild-FGF was accumulated in the liver at a higher level than M8-FGF or M5-FGF, and each of Wild-FGF, M8-FGF, and M5-FGF showed a slower clearance rate from the liver than CHO-derived secFGFs (i.e., the biantennary complex type and the non-glycosylated type). Since the accumulation of yeast-derived secFGFs in the liver was about 50–60% of the administered dose even 30 min after intravenous injection, Wild-, M8- and M5-FGFs were likely to be resistant to proteolysis or very slowly metabolized in the liver. This is confirmed by the data on stomach distribution, which showed that the radioactivity of yeast-derived secFGFs was about half that of CHO-derived secFGFs at 30 min after injection. The radioactivity in the stomach suggested that the released ^{125}I- or small molecules containing ^{125}I derived from ^{125}I-secFGF might accumulate in the stomach. The blood clearance of all secFGFs was very rapid and seemed to be largely affected by the hepatic uptake. However, M8- and M5-FGFs disappeared from the blood much more quickly than other secFGFs, a finding that could not be explained only by the accumulation in the liver. The concentrations of M8- and M5-FGFs in the kidney were also much higher than those in the blood, and this phenomenon was not observed for Wild-FGF. It is known that the limiting size of glomerular filtration is dependent on the pore size of the glomerular basement membrane [30]. Since Kinoshita *et al.* [31] have demonstrated that deglycosylation of human erythropoietin accelerates its filtration out into the urine, we think that the size of the carbohydrate moiety is important. The clearance rate of yeast products from the blood was very rapid, but glomerular filtration was not likely to be responsible for this, because the difference in sugar chain size between the complex biantennary type (CHO product) and the high-mannose type (mutant yeast product) was not significant. It was more likely that M8- and M5-FGFs were recognized by a high-mannose-specific receptor expressed in the kidney, rather than by a mannose receptor. Generally, in the clearance of soluble ligands by a mannose receptor, the ligand is sent to the lysosomes for degradation [32]. However, a receptor specifically recognizing high-mannose type sugar chains may exist in the kidney to regulate some of the autocrine factors, and other factors as well. Feige *et al*. showed that FGF receptors were expressed in kidney cells and were glycosylated to high-mannose type and complex-type sugar chains, suggesting that

the carbohydrate contributes to receptor function [33]. It is also well known that FGF is secreted from kidney cells [34]. Unfortunately, the interaction between the carbohydrate of FGF and the FGF receptor is not clear at present. Further studies will be needed to determine whether this kidney receptor that specifically recognizes high-mannose type sugar chains can be utilized for targeting therapy. Here, we established a mutant yeast strain for production of high-mannose type glycoform protein, and demonstrated a specific accumulation of M8- and M5-FGFs in the kidney. It is known that glycoproteins expressed in insect (Sf9) cells contain only high-mannose type carbohydrate chains [35]. However, yeast has some advantages over insect cells, in terms of the ease of large-scale cultivation and the simplicity of gene manipulations, including gene targeting. Recently, Chiba *et al*. [36] succeeded in producing α-galactosidase A with mannose-6-phosphate residues in yeast. The sugar chain moiety of the recombinant enzyme was made by utilization of the unique carbohydrate synthetic pathway of the yeast, and the enzyme may be useful in enzyme-replacement therapy for patients with Fabry disease. We believe that yeast is a suitable host cell for producing various remodeled glycoproteins for tissue-targeting therapy.

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