General and Comparative Endocrinology 276 (2019) 37–44

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Site-specific roles of *N*-linked oligosaccharides in recombinant eel follicle-stimulating hormone for secretion and signal transduction

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ARTICLE INFO

Keywords:
Japanese eel
Follicle-stimulating hormone
N-linked oligosaccharide
cAMP responses
EelFSH receptor

ABSTRACT

Eel follicle-stimulating hormone (eelFSH) is composed of a common α-subunit and a hormone specific β-subunit, both of which contain two *N*-linked carbohydrate residues. We characterized the biologically active single chains by fusing the α-subunit to the carboxyl terminal region of the eelFSH β-subunit. Expression vectors were constructed and the biological activity of the recombinant hormones (rec-hormones) was characterized using Chinese hamster ovary (CHO) K1 cells expressing the eelFSH receptor gene. Mutagenesis of the individual and double glycosylated sites was performed to determine the functions of the oligosaccharide chains on signal transduction. The absence of the Asn^{22} (eelFSHβΔ22/α) and $Asn^{5.22}$ (eelFSHβΔ5.22/α) *N*-linked oligosaccharide chain in the eelFSH β-subunit completely reduced the secretion level in the medium and cell lysate of CHO-K1 cells. The expression levels of eelFSHβ/α wild-type in CHO suspension (CHO-S) cells was approximately 4-fold higher in CHO-k1 cells. The molecular weight of rec-eelFSHβ/α wild-type by western blotting analysis was found to be 34 kDa. Mutants (β/αΔ56, β/αΔ79, and βΔ5/α) lacking single oligosaccharide sites showed molecular weights that were reduced by approximately 10%. The digestion of N-linked oligosaccharides using PNGaseF treatment showed that the molecular weights of all mutants were reduced to 27-kDa. The oligosaccharide chains in rec-eelFSHβ/α wild-type were modified to a molecular weight of approximately 7–10 kDa in CHO-K1 and CHO-S cells. Oligosaccharide site deletions at positions Asn^{56} and Asn^{79} on the α-subunit and Asn^{5} on the β-subunit were found to play an essential role in cAMP signal transduction through the eelFSH receptor. The EC_{50} values of Asn^{56} and Asn^{5} resulted in a significant decrease in potency to 64% and 53% of the wild type, respectively. Specifically, the removal of the carbohydrates at Asn^{79} of the α-subunit (β/αΔ79) was drastically reduced to 53.8% of the wild-type levels in maximum response. These results have allowed for the identification of the site-specific roles of carbohydrate residues in eel FSH. Our data suggest that *N*-linked oligosaccharide chains play a pivotal role in biological activity through the eelFSH receptor as suggested in similar studies of other mammalian FSH hormones.

1. Introduction

Follicle stimulating hormone (FSH) is a member of the glycoprotein hormone family, alongside luteinizing hormone (LH), thyrotropin stimulating hormone (TSH), and human chorionic gonadotropin (hCG). These hormones are composed of a common α-subunit and a unique β-subunit (Min et al., 2004). Gonadotropin hormone synthesis and secretion from the pituitary involve the integrated actions of hypothalamic gonadotropin-releasing hormone (GnRH) and gonadal hormones (Muyan et al., 1994). Although the release of LH and FSH overlap at the preovulatory surge, the secretions diverge under a variety of physiologies.

Site-directed mutagenesis can be used to examine the role of individual glycosylation sites by removing oligosaccharide attachment. Specifically, the *N*-linked oligosaccharide chains on the α-subunit at position 52 are important for hCG, human FSH (hFSH), and human TSH (hTSH) signal transduction (Bishop et al., 1994; Fares et al., 1996; Flack et al., 1994; Valove et al., 1994). Several studies have shown that

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https://doi.org/10.1016/j.ygcen.2019.03.003
Received 7 August 2018; Received in revised form 27 February 2019; Accepted 1 March 2019
Available online 02 March 2019

Table 1
List of primers used to construct the eelFSH mutants.

	Primer name	Location	Primer Sequence
1	eelFSH*β/α* forward	–	5′-TGAATTCATGCATCTGGCTGTCAC-3′ *Eco*R I site
2	eelFSH*β/α* reverse	–	5′-ACCTCGAGTTAAAATTTGTGGTAGTAGCA-3′ *Xho* I site
3	eelFSH*β/α56* forward	*α* 56	5′-ATGCTGGTGCCAAAGCAGATTACATCTGATGCA-3′
4	eelFSH*β/α56* reverse	*α* 56	5′-TGCCTCAGATGTAATCTGCTTTGGCACCAGCAT-3′
5	eelFSH*β/α79* forward	*α* 79	5′-AACATGAAACTGGAGCAGCACACAGACTGCCAC-3′
6	eelFSH*β/α79* reverse	*α* 79	5′-GTGGCAGTCTGTGTGCTGCTCCAGTTTCATGTT-3′
7	eelFSH*β5/α* forward	*β* 5	5′-TGTGGCTCGCCCAGATCTCCATCTCCGTG-3′
8	eelFSH*β5/α* reverse	*β* 5	5′-CACGGAGATGGAGATCTGGGCGAGACCACA-3′
9	eelFSH*β22/α* forward	*β* 22	5′-GGCTGCATCACCTTCCAGACCACCGCCTGTGCT-3′
10	eellFSH*β22/α* reverse	*β* 22	5′-AGCACAGGCGGTGGTTCTGGAAGGGTGATGCAGCC-3́

the removal of carbohydrate residues from hFSH greatly decreased the biological activity (Bishop et al., 1994; Flack et al., 1994; Valove et al., 1994). Thus, the oligosaccharides have been shown to play a role in determining the biological activity of FSH (Sairam and Bhargavi, 1985) and plasma half-life (Sebok et al., 1990; Sairam, 1989). Biosynthesis of a biologically active single peptide chain was first suggested in the hCG (Sugahara et al., 1995), hLH (Garci-Campayo et al., 1997), hFSH (Sugahara et al., 1996a,b), and hTSH (Fares et al., 1996). The single chain exhibited remarkably greater *in vitro* stability than the heterodimer (Garci-Campayo et al., 1997). We also reported that the biological activities of equine CG (eCG) and eFSH single chains are higher than that of wild type (Min et al., 2004; Park et al., 2017; Lee et al., 2007).

The Japanese eel, *Anguilla japonica*, is one of the major aquaculture species in East Asia (Ohta et al., 2017). In the eel, single-chain gonadotropin hormones (GTHs) of rec-eelFSH and -eelLH were reported to exhibit biological activity (Kobayashi et al., 2010; Ohta et al., 2007). rec-eelFSH stimulated both testosterone and estradiol-17β secretion in a dose-dependent manner from the ovary with fully developed theca and granulosa cells around mid-vitellogenic oocytes (Kamei et al., 2006). A large quantity of Japanese eel rec-FSH and rec-LH was produced using a *Drosophila* expression system and the biochemical properties of these rec-GTHs were reported (Kazeto et al., 2008). Recently, rec-eel GTHs were produced from cell lines of Chinese hamster ovaries (CHO) and these rec-hormones have shown more effective results than conventional salmon pituitary extracts for the induction of oogenesis and spermatogenesis (Kazeto et al., 2014). The amount of expressible milt rapidly increased after 12 h by rec-eelLH injection in mature male eels. Sperm motility (%) and velocity also showed a sharp rise after rec-eelLH injection, peaking at 12 h and maintaining high values until 42 h (Ohta et al., 2017). We recently produced specific monoclonal antibodies for eelFSH and developed a sandwich enzyme-linked immunosorbent assay (ELISA) system for the analysis of rec-eelFSH (Kim et al., 2016a,b). We also reported that the signal transduction activity of equine CG (eCG) and eFSH lacking the oligosaccharide at Asn^{56} of the α-subunit was markedly reduced (Min et al., 1996, 2004; Park et al., 2017; Saneyoshi et al., 2001). Recently, we also reported that the absence of carbohydrates at Asn^{56} of the α-subunit and at Asn^{10} of the β-subunit in rec-eelLH plays a pivotal role in cAMP biopotency using cells expressing the eelLH receptor gene (Byambaragchaa et al., 2018).

The eel FSH α-subunit has two asparagine-linked (Asn-linked and/or *N*-linked) carbohydrate chains present at positions 56 and 79. The β-subunit also contains two Asn-linked chains at positions 5 and 22 (Kim et al., 2016b). To our knowledge, the roles of glycosylated sites have not been identified in the *Anguilla japonica* eelFSH gene. In order to identify and define the roles of the carbohydrate residues, we mutated Asn with the glutamate (Gln) codon and expressed the mutated cDNAs in CHO-K1 cells and CHO-suspension cells. Determination of the biological activity of these variants was carried out for the identification of specific glycosylated sites that are essential for signal transduction through eelFSH receptor. We also are going to utilize rec-FSH to understand the regulatory mechanisms of gonadal development and to succeed in the artificial induction of gonadal maturation.

2. Materials and methods

2.1. Materials

The oligonucleotides used in this study were synthesized by Genotech (Daejon, Korea). The cloning vector, pGEMTeasy, was purchased from Promega (Madison, WI, USA). The pcDNA3 mammalian expression vector, CHO-suspension (CHO-S) cells, FreeStyle MAX transfection reagents, and Lipofectamine-2000 were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Ham's F-12 medium, OptiMEM medium, and CHO-S-SFMII medium were purchased from Gibco BRL (Grand Island, NY, USA). Monoclonal antibodies, 5A11 anti eelFSH α, 5A14 anti eelFSHβ, and horseradish peroxidase-conjugated anti eelFSH 11A8, were produced in our lab as previously described (Kim et al., 2016a,b). The cAMP HTRF assay kit was purchased from Cisbio (Codolet, France). The deglycosylation kit (PNGase) was purchased from New England Biolabs (Ipswich, MA, USA). Endonucleases and polymerase chain reaction (PCR) reagents were obtained from Takara (Shiga, Japan). The Lumi-Light western blot kit was obtained from Roche (Basel, Switzerland). The QIAprep-Spin plasmid kit was purchased from QIAGEN Inc. (Hilden, Germany). Fetal bovine serum was obtained from Hyclone laboratories (Logan, UT, USA) and disposable spinner flasks were obtained from Corning Inc. (Corning, NY, USA). Centriplus Centrifugal Filter Devices were purchased from Amicon Bio separations (Billerica, MA, USA). All other reagents used were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Wako Pure Chemicals (Osaka, Japan).

2.2. Site directed mutagenesis

For site-directed mutagenesis, an overlap extension PCR strategy was used as previously reported (Min et al., 2004). Primer sequences are shown in Table 1. The glycosylation sites with asparagine (AAC) were replaced with the codons for glutamine (CAG) at the amino acid positions of the α-subunits 56 and 79 and the β-subunit 5 and 22. The strategy for wild-type and mutant subunits is shown in Fig. 1. An individual mutant (αΔ56) was used as a template and mutant primers for another individual mutant (αΔ79) were used for the PCR amplification to generate the double mutant (αΔ56.79). In the eel FSHβ-subunit, mutagenesis for two glycosylated sites (βΔ5 and βΔ22) were amplified and used to generate the double mutant (βΔ5.22). The newly synthesized full-length PCR product was eluted and cloned into a pGEMTeasy vector. The clone was used to transform DH5 α competent cells. Plasmids were isolated and sequenced to check for PCR errors.

2.3. Vector construction

cDNA encoding the eelFSHβ/α α and deglycosylated mutants was

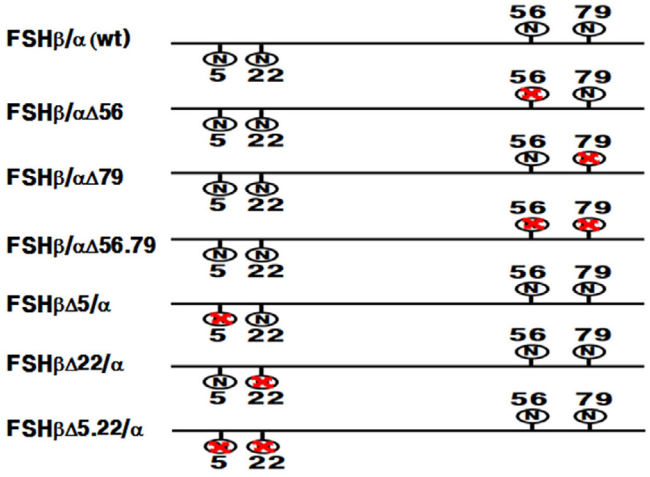

Fig. 1. Schematic diagram of rec-eelFSHβ/α mutants. Wild-type and mutants with altered *N*-linked oligosaccharide sites on eelFSH are shown. The Asn^{56} and Asn^{79} codons of the α-subunit and the Asn^{5} and Asn^{22} of the β-subunit were replaced with Gln by PCR site-directed mutagenesis as previously described (Min et al., 2004). A circle "N" denotes an *N*-linked oligosaccharide, while "X" denotes the absence of an oligosaccharide. Seven expression vectors were constructed by insertion into the *Eco*RI and *Xho*I sites of the pcDNA3 mammalian expression vector (designated as pcDNA3-eelFSHβ/α; β/αΔ56; β/αΔ79; β/αΔ56.79; βΔ5/α, βΔ22/α, and βΔ5.22/α, respectively).

inserted into the pcDNA3 mammalian expression vector as previously reported (Min et al., 2004). These fragments were digested with *Eco*RI and *Xho*I, and ligated into the eukaryotic expression vector, pcDNA3. We also constructed each mutant for removal of four *N*-linked oligosaccharide chains at positions Asn^{56} and Asn^{79} of the α-subunit and Asn^{5} and Asn^{22} of the β-subunit as previously reported (Byambaragchaa et al., 2018). The plasmids were then purified and sequenced in both directions by automated DNA sequencing to ensure that the correct mutations had been introduced. Seven expression vectors were constructed (designated as pcDNA3-eelFSHβ/α, eelFSHβ/α Δ56, eelFSHβ/α Δ79, eelFSHβ/α Δ56.79, eelFSHβΔ5/α, eelFSHβΔ22/α, and eelFSHβΔ5.22/α).

2.4. Transient transfection

The expressing vectors were transfected into the cultured CHO-K1 cells with expression vectors using the liposome transfection method, as previously described (Min et al., 2004). The transfected culture medium was exchanged with a serum-free medium (CHO S-SFMII) at 24 h after transfection. The culture supernatants were collected at 72 h after transfection. The supernatant was stored at −20 °C until the assay. In the CHO-S cells, plasmid vectors were transfected by FreeStyle MAX reagent according to the manufacturer's instructions. One day prior to transfection, CHO-S cells were passaged at 5×10^5 cells/mL. Flasks were placed on an orbital shaker platform rotating at 120–135 rpm at 37 °C in a humidified atmosphere of 8% CO_2 in air. On the day of transfection, 80 μg of plasmid DNA was diluted into OptiPRO™ serum-free medium (SFM) to a total volume of 1.2 mL. In a separate tube, 80 μL of FreeStyle™ MAX Reagent was diluted in Opti-Pro™ SFM to a total volume of 1.2 mL and mixed gently by inverting the tube. After the solutions were mixed, DNA-FreeStyle™ MAX Reagent complex was slowly added to 60 mL medium containing cells. For the rec-protein assay, 1 mL culture medium was collected on days 1, 3, 4, 5, 6, and 7. Finally, the culture media were collected on day 7 after transfection and centrifuged at 100,000 × g at 4 °C for 10 min to remove cell debris. Supernatants were collected and frozen at −80 °C. The samples were concentrated by freeze-drying and mixed with PBS. rec-proteins were analyzed by western blotting or enzyme-linked immunosorbent assay (ELISA).

2.5. Quantification of rec-eelFSH proteins

rec-eelFSH wild type and mutant hormones in cell-culture media were quantified by a double-sandwich ELISA method using plates coated with the monoclonal antibody eelFSH 5A11 directed against the α-subunit of eelFSH as previously described by our lab (Kim et al., 2016a,b). The wells were blocked by incubation with 1% skimmed milk in phosphate buffered saline (PBS) for 1 h at 37 °C. The wells were then washed with filtered PBS containing 0.05% Tween 20 (PBS-T). Next, 100 μL of rec-eelFSH hormone samples and standard samples were added to the wells and incubated for 1–2 h at 37 °C. After washing, horseradish peroxidase-conjugated anti eelFSH 11A8 (binds α-subunit of eelFSH) antibody was added and incubated for 1 h at room temperature. The substrate solution (100 μL) was added, and the mixture was then incubated for 20 min at room temperature. The reaction was stopped by adding 50 μL of 1 M H_2SO_4. The optical density (OD) of the product solution was read at 450 nm with a microplate reader (Cytation 3, Biotek, Winooski, VT, USA). For this assay, purified rec-eelFSHβ/α protein, previously produced in our lab, was used as a standard at concentrations of 0–400 ng/mL.

2.6. Detection of rec-eelFSHs by western blotting and enzymatic digestion of N-linked oligosaccharides

The cells were collected at 72 h after transfection. The cells were centrifuged at 100,000 × g at 4 °C for 10 min, and the cells debris was recovered. The samples were then homogenized in 600 μL PRO-PREP protein extraction solutions. Cell lysis was induced by incubating cells for 30 min on ice and the supernatants were collected by centrifuged at 100,000 × g at 4 °C for 10 min.

For western blotting analysis, the concentrated samples (15 μg) from cells or media were electrophoresed under reducing condition on 12.5% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) (Laemmi, 1970). The protein was transferred to a polyvinyli-dene difluoride (PVDF) membrane (0.2 μm) using a Mini Trans-Blot electrophoretic transfer cell. After blotting, the membrane was incubated with monoclonal anti-eelFSH α antibody (5A14) for 13–15 h. The blot was then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody for 2 h. Thereafter, the membrane was incubated for 5 min with 2 mL Lumi-Light substrate solution, and detection was performed using the enhanced chemiluminescence system.

The rec-eelFSH proteins were analyzed to remove glycans added by the *N*-glycosylation enzyme. For removal of all *N*-linked glycans, rec-eelFSH protein (15 μg) was incubated for 1 h at 37 °C with PNase F [1 μL enzymes (2.5 U/mL)/20 μL sample + 2 μL of 10 × Glycobuffer + 2 μL of 10% NP-40]. The reaction was stopped by boiling for 10 min with 2 μL of 10 × Glycoprotein Denaturing Buffer. The samples were electrophoresed by SDS-PAGE and analyzed by western blotting.

2.7. cAMP assay via homogenous time-resolved foster resonance energy transfer (HTRF)

Measurement of cAMP accumulation in CHO-K1 cells expressing eelFSHR was performed using cAMP Dynamic 2 competitive immunoassay kits (Cisbio Bioassays, Codolet, France) as described previously (Byambaragchaa et al., 2018). Briefly, the cAMPs assay used a cryptate-conjugated anti-cAMP monoclonal antibody and d2-labeled cAMP. Cells transfected with eelFSHR were added at 10,000 cells per well into a 384-well plate. rec-eelFSHβ/α-WT and mutant proteins were added and incubated for 30 min. The assay was terminated by the addition of Cisbio detection reagents, cAMP-d2, and anti-cAMP-cryptate (5-fold diluted in lysis buffer and used at 5 μL/well), and the samples were incubated for 1 h at room temperature. cAMP was detected by measuring the decrease in HTRF energy transfer (665 nm/620 nm)

using an Artemis K-101 HTRF Microplate reader (Kyoritsu Radio, Tokyo, Japan). The method was a competitive immunoassay between native cAMP produced by cells and cAMP labeled with the dye d2. Tracer binding was visualized using a Mab anti-cAMP antibody labeled with Eu3 + Cryptate. The specific signal-Delta F (energy transfer) was inversely proportional to the concentration of cAMP in the standard or sample. Results were calculated from the 665 nm/620 nm ratio and expressed as Delta F % (cAMP inhibition).

Delta F% = (standard or sample ratio − sample negative)
× 100/ratio negative

The cAMP concentration for Delta F% values were calculated using GraphPad Prism software (GraphPad, Inc., La Jolla, CA, USA).

2.8. Data analysis

The Multalin interface-multiple sequence alignment tool was used for sequence analysis and comparisons; the GraphPad Prism 6.0 (GraphPad, Inc) was used for cAMP production analysis and cAMP EC_{50} and Grafit 5.0 (Erithacus Software Limited, Surrey, UK) was used for stimulation curve analysis. Curves fitted in a single experiment were normalized to the background signal measured for mock-transfected cells (0%). Each curve was drawn using data from at least three independent experiments. One-way ANOVA Tukey's Multiple Comparison tests were used to compare the results between samples, using GraphPad Prism 6.0. Differences were indicated as significant between the groups ($P < 0.05$).

3. Results

3.1. Secretion of rec-eelFSH proteins into CHO-cells

Site-directed mutagenesis was carried out to examine the functional effects of glycosylated sites on rec-eelFSH biological activity. We constructed seven expression vectors including the eelFSHβ/α (wild type) as shown in Fig. 1. In order to analyze the secreted quantity into the cell medium, the expression vectors were transiently transfected into CHO-K1 cells. The concentrations of rec-eelFSH protein expression for β/α, β/α Δ56, β/α Δ79, β/α Δ56.79, βΔ5/α, βΔ22/α and βΔ5.22/α in CHO-K1 cells were 165.7 ± 15, 134.7 ± 7, 96.8 ± 12, 124.6 ± 2, 104.1 ± 10, 19.1 ± 17, and 40.2 ± 14 ng/mL, respectively (Fig. 2). The expression level of the deglycosylated mutants slightly decreased compared to wild type. Particularly, a large decrease in expression was observed for the rec-eelFSHβΔ22/α and rec-eelFSHβΔ5.22/α mutants. These results indicate that the glycosylation site at Asn^{22} of the β-subunit specifically affected secretion into the medium of CHO-K1 cells. Next, we analyzed the expression level of eelFSHβ/α wild-type in the

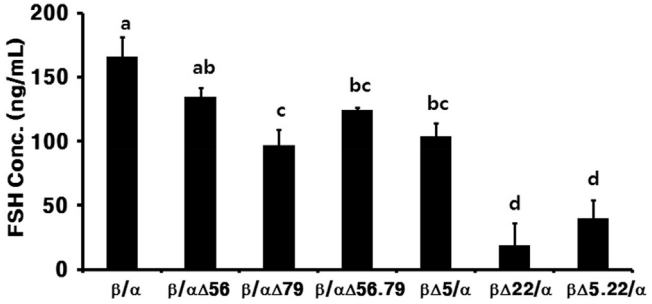

Fig. 2. Secretion levels of rec-eelFSHβ/α wild-type and mutants following transient transfection in CHO-K1 cells. The expression vectors were transfected into CHO-K1 cells and the supernatants were collected at 72 h after transfection. Expression levels of rec-eelFSH mutants were detected by ELISA as described in the Material and Methods. Values with different superscripts were significantly different ($P < 0.05$).

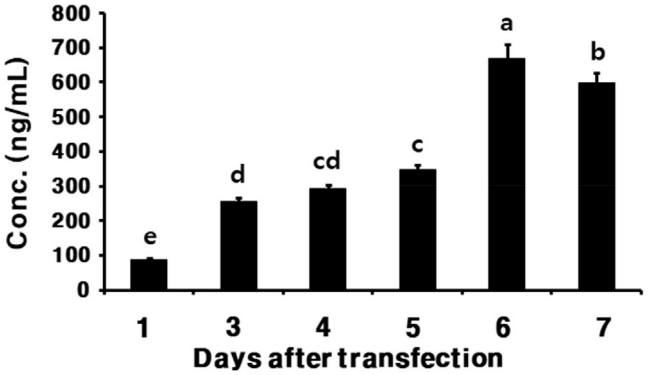

Fig. 3. Secretion levels of rec-eelFSHβ/α wild-type following transient transfection in CHO-suspension cells over time. The expression vectors were transfected into CHO-suspension cells and the supernatants were collected every day after transfection. The expression levels of rec-eelFSHβ/α wild-type were analyzed with sandwich ELISA in triplicate independent repeats as described in the Material and Methods. Values with different superscripts were significantly different ($P < 0.05$).

FreeStyle MAX-CHO-suspension (CHO-S) expression system, which was 257.7 ± 10 ng/mL at 3 days after transfection, subsequently increasing until 6 days at which point the level was approximately 670.2 ± 40 ng/mL. After then it was a little decreased to 598.5 ± 30 ng/mL at 7 days (Fig. 3). The expression levels of eelFSHβ/α wild-type in CHO-S cell was approximately 4-fold higher than that of CHO-K1 cells.

3.2. Western blotting analysis and deglycosylation by PNGase F

Next, the molecular weight of the rec-eelFSH mutants was determined by western blotting using the anti-eelFSH5A14 monoclonal antibody. Western blot analysis revealed an approximate molecular weight of 34 kDa for rec-eelFSHβ/α produced from CHO-K1 cells as shown in Fig. 4. Rec-eelFSHβ/αΔ56, β/αΔ79, and βΔ5/α, without one *N*-linked oligosaccharide, were reduced to approximately 31 kDa, showing a decrease of 3 kDa in molecular weight. The molecular weight of the double mutant (rec-eelLHβ/αΔ56.79) was 28 kDa, showing an approximately 6 kDa decrease as shown in Fig. 4A. In the cell lysate, we also performed the western blotting. As shown in Fig. 4B, we did not detect any signal in the deglycosylated mutant of β-subunit^{22} and double mutant of β-subunit$^{5.22}$. Although the expression protein was detected by ELISA analysis, we did not find any bands in the βΔ22/α and βΔ5.22/α mutants. The absence of and Asn^{22} N-linked oligosaccharide chain in the eelFSH β-subunit had a significant effect on the expression quantity.

Next, we produced all mutants in the CHO-S expression system and concentrated the samples by 20-fold by freeze-drying and using Centriplus centrifugal filters. The molecular weight was detected similar to CHO-K1 cells (Fig. 5A). To further characterize the N-linked oligosaccharides, we digested rec-eelFSH proteins produced from CHO-S cells with PNGase F. This enzyme cleaves high-mannose incompletely processed oligosaccharides. All rec-proteins decreased to approximately 27 kDa in molecular weight (Fig. 5B), demonstrating the removal of two oligosaccharide chains from αΔ56.79 and one chain from αΔ56, αΔ79, and βΔ5. However, it is difficult to determine the precise molecular mass for carbohydrate content on SDS gels (Fig. 5).

3.3. Biological activity of rec-eelFSH mutants

The *in vitro* biological activity of the mutants was assessed by using transfected CHO-K1 cells expressing the eelFSH receptor. The cells were incubated with conditioned medium from transfected CHO-K1 cells expressing eelFSH mutants. The potency of cAMP activation for rec-

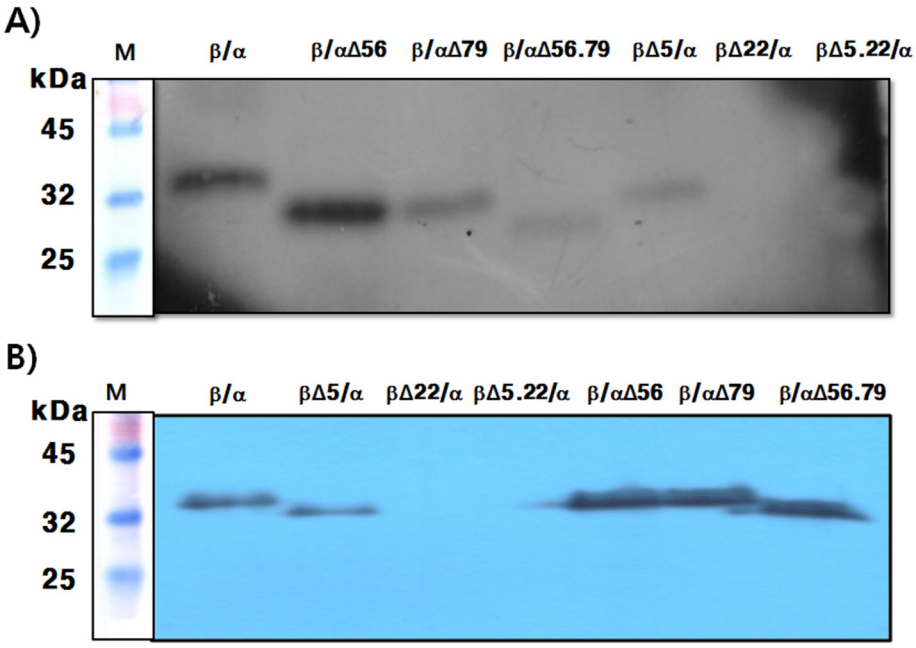

Fig. 4. Western blot analysis of rec-eelFSHβ/α mutants. rec-eelFSHβ/α proteins including mutants lacking glycosylation sites were expressed in CHO-K1 cells. The proteins in conditioned media (A) and cell lysate (B) were collected, concentrated, separated by SDS-PAGE, and transferred to a blotting membrane. The proteins were detected with a monoclonal antibody (anti-eelFSH5A14) against the eel FSH β-subunit.

eelFSH mutants is shown in Fig. 6. The dose-response curves of all deglycosylated mutants were notably shifted to the right (Fig. 6). The rec-eelFSHβ/α wild-type exhibited full biological activity, suggesting that the EC_{50} value was 78.6 ± 4.0 ng and R max was 112.4 ± 4.6 nM/10^4 cells. A comparison of the rec-eelFSH wild-type and the mutants confirmed the site specificity of the oligosaccharide chains at residue 56 of the α-subunit and residue 5 of the β-subunit (Fig. 6). This is clearly reflected by the significantly lower determined bioactivity of both β/αΔ56 and βΔ5/α. The EC_{50} values resulted in a significant decrease in potency to 64% and 53% of the wild type, respectively. Although the EC_{50} value of β/αΔ79 was approximately 1.2-fold (126%) higher than that of the wild type, it only reached 53% of the maximal stimulation achieved by the wild type molecule (Table 2). The removal of both oligosaccharide chains from the α-subunit (β/αΔ56.79) resulted in a 73% bioactivity compared to the wild-type. The maximum stimulation by the double mutant was approximately 78% of that observed for the fully glycosylated wild-type hormone. These rec-eelFSH glycosylated sites are critical for signal transduction through the eelFSH receptor. As a result, sites 56 and 79 of the α-subunit and 5 of the β-subunit play a pivotal role in signal transduction.

3.4. Discussion

Within the same species, all four glycoprotein hormones contain a common α-subunit and a hormone-specific β-subunit. The variety of oligosaccharide structures found on glycoproteins results from the action of many processing enzymes in the secretory pathway (Bielinska et al., 1989). The oligosaccharides at the same glycosylation site can be processed differently in tissues (Parekh et al., 1987). The importance for *N*-linked oligosaccharides processing has been discussed previously in many studies that are critical for efficient secretion and assembly of heterodimeric glycoproteins and are important for proper folding of the subunit (Hubbard, 1987; Fares, 2006; Sairam and Bhargavi, 1985). In the dimeric human FSH, the carbohydrate residue at position 52 on the α-subunit was reported to play an essential role in signal transduction as its removal resulted in a significant decrease in potency to 26% of

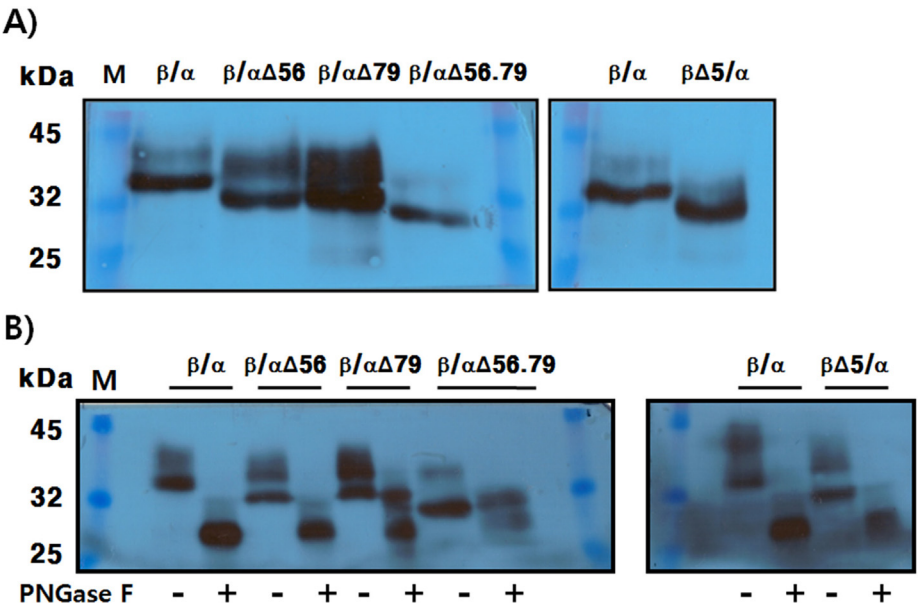

Fig. 5. Western blot analysis of rec-eelFSHβ/α mutants expressed in CHO-suspension cells. (A) rec-eelFSHβ/α proteins were expressed in CHO-suspension cells. The proteins in conditioned media were collected, concentrated, separated by SDS-PAGE, and transferred to a blotting membrane. The proteins were detected with a monoclonal antibody (anti-eelFSH5A14) against the eel FSH β-subunit (A). Proteins for western blotting were also treated with *N*-glycosidase-F (B). PNGase digestion was conducted for 1 h at 37 °C and proteins were separated by SDS-PAGE. Lane 1: β/α; Lane 2: β/αΔ56; Lane 3: β/αΔ79; Lane 4: β/αΔ56.79; Lane 5: βΔ5/α. : non-treated; +: treated with *N*-glycosidase-F.

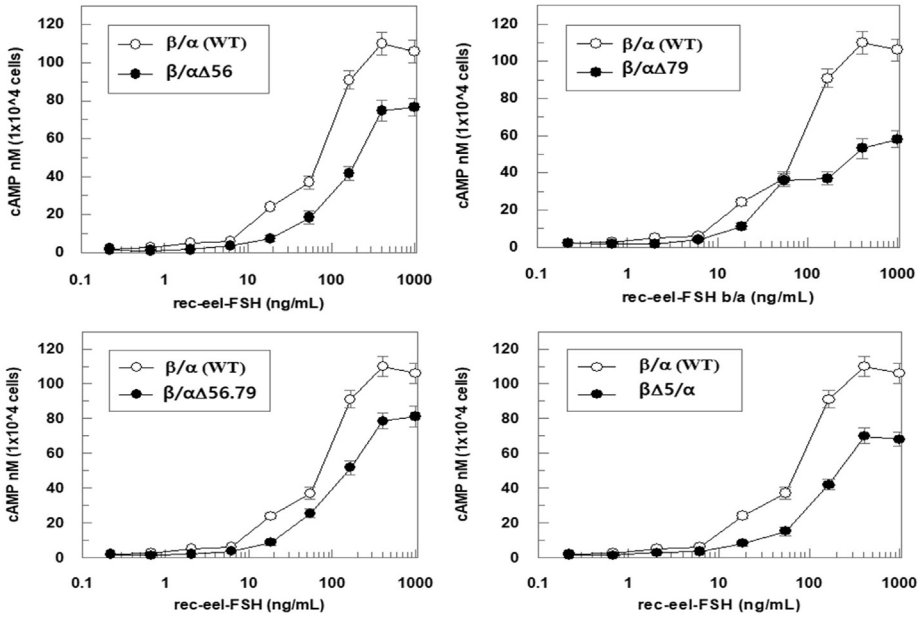

Fig. 6. Dose-dependent increase in total cAMP accumulation induced by rec-eelFSHβ/α wild-type and deglycosylated mutants in cells expressing eelFSH receptor. Cells expressing the eelFSH receptor were added at 10,000 cells per well into a 384-well plates. Standard samples were prepared at 0.17–712 nM. The plate was incubated for 30 min at room temperature after the addition of rec-eelFSHβ/α proteins (0–1000 ng/mL). The inhibition of cAMP accumulation is shown as Delta F%. cAMP concentration (1×10^4 cells) was calculated using GraphPad Prism software.

Table 2
Bioactivity of the rec-eelFSH β/α wild-type and deglycosylated mutants.

rec-eelFSHβ/α mutants	cAMP responses		
	Basal (nM/10^4 cells)	EC_{50} (ng)	Rmax (nM/10^4 cells)
rec-eelFSHβ/α (wild type)	4.9 ± 0.5	78.6 ± 4.0 (100%)	112.4 ± 6.6
rec-eelFSHβ/αΔ56	2.3 ± 0.1	152.1 ± 5.3 (64%)	83.8 ± 5.9
rec-eelFSHβ/αΔ79	1.9 ± 0.2	62.1 ± 2.5 (126%)	60.5 ± 8.5
rec-eelFSHβ/αΔ56.79	1.8 ± 0.3	118.0 ± 5.2 (73%)	87.8 ± 3.9
rec-eelFSHβΔ5/α	2.9 ± 0.3	134.8 ± 4.0 (53%)	72.8 ± 4.1

Values are the means ± SEM of triplicate experiments. The EC_{50} values used to determine the potencies were determined from the concentration–response curves for the *in vitro* bioassays.

wild type levels (Bishop et al., 1994). The Asn^{52} of the α-subunit also has a disproportionate role in signal transduction, and the amino acid sequence at Asn^{24} of the β-subunit functions in both binding and signal transduction (Flack et al., 1994). Single-chain gonadotropins, which fused the α-subunit to the carboxyl terminal region of the FSH β-subunit, were secreted efficiently and are biologically active (Saneyoshi et al., 2001; Sugahara et al., 1996a,b). However, there are no reports on this except for our recent research on the functions of the gonadotropin oligosaccharide of rec-eel LH in eel (Byambaragchaa et al., 2018).

In this work, we characterized each of the four glycosylation sites in the secretion and signal transduction of the eel FSH α-subunit and β-subunit. Here, we prepared deglycosylated mutants (β/αΔ56, β/αΔ79, β/αΔ56.79, βΔ5/α, βΔ22/α and βΔ5.22/α) to investigate the biological functions of oligosaccharide chains in single-chain eelFSH by site-directed mutagenesis. The present study indicated that a single chain of eel FSH exhibited full biological activity and that *N*-linked oligosaccharide chains play a pivotal role in determining biological activity through the eelFSH receptor. The absence of the Asn^{22} of the β-subunit was greatly decreased and resulted in the poor secretion. This is consistent with the loss of the oligosaccharide at position 78 of the hCG α-subunit, which caused the mutant subunit to be degraded quickly and only < 20% was secreted (Matzuk and Boime, 1988a). However, we found that the rec-eelLH β/αΔ79 mutant was only slightly decreased and that the other deglycosylated mutants did not have an effect on secretion into the medium (Byambaragchaa et al., 2018). The specific glycosylated sites (Asn^{82} of α-subunit and Asn^{13} of β-subunit) are very important in secretion of the rec-equine CG (rec-eCG) dimer and single chain (in preparation). Thus, we suggest that the each of the glycosylation sites has a different effect on secretion among mammalian and fish species.

The yield of rec-eelFSHβ/α was approximately 0.67 mg/L, which reached a maximum after 6 days of transfection in the transient transfection using CHO-suspension cells. According to previous reports, the yield of rec-eelFSHβ/α was 2.0 mg/L using methylotrophic yeast, *Pichia pastoris* (Kamei et al., 2003), obtaining 2.2 mg from 250 infected larvae (Kobayashi et al., 2010) and 3.8 mg/L in *Drosophila S2* cells (Kazeto et al., 2008). The production of catfish rec-FSHβ/α yielded 7 mg/L in the same expression system (Zmora et al., 2007). Thus, a stable single cell line expressing a higher quantity should be isolated to induce for *in vivo* eel maturation.

In the eel pituitary, the purified eel α-subunit specifically reacted to the 19 kDa protein. The FSH β-subunit was detected two bands between 21 and minor 17 kDa. After *N*-glycopeptidase F treatment, the molecular weight of the eel α-subunit and the FSH β-subunit decreased to 13 kDa and 15 kDa, respectively. The 17 kDa form was the equivalent to 19 kDa of α-subunit with a carbohydrate modification (Kamei et al., 2005). However, the purified rec-eelFSH α-subunits in *Drosophila* S2 cells were single peptides with an estimated molecular weight of 17.5 kDa. The rec-FSH β-subunit appeared to be made up of two glycoprotein bands (15.5 and 14 kDa) (Kazeto et al., 2008). In methylotrophic yeast, *Pichia pastoris*, the rec-eelFSH α-subunit was a 26 kDa protein and the broad immunoreactive bands in rec-eelFSH β-subunit were detected at 16.4 and 26.4 kDa (Kamei et al., 2003). Eel FSH molecular weight was determined to be approximately 30–40 kDa from the eel pituitary (Kamei et al., 2005) and a broad band ranged between approximately 39 and 49 kDa in *Pichia pastoris* (Kamei et al., 2003).

These results suggest that the expressed rec-eelFSH protein is glycosylated and some types of carbohydrate are differently modified among the cell type. In the single chain rec-eelFSH β/α, a specific broad band was detected at approximately 30–35 kDa for rec-eelFSH β/α including a 34-amino acid linker of the eCG β-subunit C-terminal region in baculovirus (Kobayashi et al., 2010). Goldfish and trout rec-FSHβ/α in silkworm larvae were detected as 38 kDa and 35 kDa, respectively (Hayakawa et al., 2008; Ko et al., 2007). These results are nearly consistent with our result, suggesting that the molecular weight of rec-eelFSHβ/α wild-type in this study was approximately 34 kDa from CHO-K1 and CHO-suspension cells. In hFSH, the single-chain FSH was secreted efficiently and was biologically active (Sugahara et al., 1996a). Efficient secretion of the FSH single chain requires a linker between the carboxy terminus of the FSH β-subunit and the amino terminus of the α-subunit (Sugahara et al., 1996b).

A previous study using rec-hCG suggested that the extracellular form of the hCG β-subunit wild-type (31 kDa) was decreased to 28 kDa because of the loss of the oligosaccharides at one site (Matzuk and Boime, 1988b). Recently, we also reported that the size of rec-eelLHβ/αΔ56, β/αΔ79, and βΔ10/α, without one *N*-linked oligosaccharide, were reduced to approximately 29 kDa, showing a decrease of 3 kDa in molecular weight. The molecular weight of the double mutant (rec-eelLHβ/αΔ56.79) was 26 kDa, showing an approximately 6 kDa decrease (Byambaragchaa et al., 2018). Our results are consistent with those of previous studies, suggesting that the molecular weights of mutants without one or two *N*-linked oligosaccharides and PNGase treatment were reduced by approximately 3–7 kDa. We confirmed that the loss of oligosaccharide chains greatly decreased the molecular weight because of the deglycosylations of the *N*-linked oligosaccharide chain of rec-eelFSHβ/α mutants. Our results indicated that the *N*-linked oligosaccharides on the α- and β-subunits were fully modified post-translation.

Many studies have reported that the biological activity of the *N*-linked oligosaccharides of each subunit alone, indicating the carbohydrates on the α-subunit of FSH are more critical in signal transduction than those on the β-subunit (Keene et al., 1994). In human FSH, the signal-transducing activity of an FSH lacking the oligosaccharide at Asn^{52} of the α-subunit was markedly reduced and that of an FSH lacking the β-subunit oligosaccharide (Asn^{7} and Asn^{24} of β-subunit) was slightly reduced (Flack et al., 1994). The oligosaccharide of Asn^{52} of the hFSH α-subunit is very important for signal transduction but not for high affinity binding (Valove et al., 1994). The signal transducing activity lacking the carbohydrate residue at Asn^{52} on the FSH α-subunit was significantly decreased to a potency of 26% of that of wild-type levels (Bishop et al., 1994). Deglycosylation of the hCG and hTSH α-subunit at Asn^{52} significantly reduced cAMP formation (Fares et al., 1996; Matzuk et al., 1989). Our recent findings, based on the site-directed mutagenesis of eelLH oligosaccharide sites, identified the Asn^{56} of the α-subunit and the Asn^{10} of the β-subunit oligosaccharides as having a major role in signal transduction (Byambaragchaa et al., 2018).

In the present study, we also found that the oligosaccharide chains at position 56 and 79 of the α-subunit and position 5 of the β-subunit play critical roles in eelFSH signal transduction. The oligosaccharide structure of rec-hFSH affects expression of genes encoding proteins, growth factors, and hormones essential for granulosa cells function (Loreti et al., 2013). We also reported that equine FSH lacking Asn^{56} of the α-subunit is indispensable for biological activity of equine FSH (Saneyoshi et al., 2001). The removal of both carbohydrate residues from Asn^{7} and Asn^{24} of the hFSH β-subunit resulted in significantly increased bioactivity (Bishop et al., 1994). In the present study, the double mutant of Asn^{56} and Asn^{79} of the α-subunit dramatically decreased in the bioactivity of EC_{50} value and maximum cAMP stimulation.

As previously suggest, the *N*-linked oligosaccharide chains in eelFSHβ/α were necessary for signal transduction. However, hypo-glycosylated $hFSH^{21/18}$ was 9- to 26- fold more active than fully-glycosylated $hFSH^{24}$ in FSH radioligand assays (Bousfield et al., 2014). The *N*-linked oligosaccharides are not important for receptor binding, but are critical for the bioactivity of glycoprotein hormones (Fares, 2006). These studies have allowed for the identification of the site-specific roles of the carbohydrate sites of eel FSH. Thus, we insist that the carbohydrate residues of rec-eelFSH are critical for efficient secretion and likely to be important for bioactivity. Our studies suggest that carbohydrate residues play an important role in determining the biological activity of eel FSH.

In conclusion, the deletion of either one of the oligosaccharide chains on the α-subunit or β-subunit decreased the biological activity of eelFSH. Our approach enabled us to identify the site-specific differences in the roles of *N*-linked oligosaccharides of eelFSH and we thus showed that the oligosaccharides at the Asn^{56} of the α-subunit and the Asn^{5} of the β-subunit play a major role in the signal transduction of eelFSH. Furthermore, there may be additional sites, such as Asn^{79} of the α-subunit, that play a pivotal role in signal transduction. Taken together, these mutants may now be useful to further analyze eel FSH interactions at eel FSH receptor *in vivo*. New rec-eelFSH mutants with high biological activity may be developed to induce eel maturation *in vivo*.

Acknowledgements

This work was financially supported by the Korean Research Foundation Program (2018R1A2B6007794), and the National Institute of Fisheries Science (R2018003), Republic of Korea.

Author contributions

J-M. K, O. M and M.B performed the experiment. B-I. L and S-K. K was financially support. M-H.K, D-J.K and K-S.M conceived the study, designed the experiment and drafted the manuscript. All authors read and approved the final manuscript.

Additional information

Competing Interests: The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.03.003.

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