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Extracellular Loop 3 (EL3) and EL3-Proximal Transmembrane Helix 7 of the Mammalian Type I and Type II Gonadotropin-Releasing Hormone (GnRH) Receptors Determine Differential Ligand Selectivity to GnRH-I and GnRH-II

Jian Hua Li, Han Choe, Ai Fen Wang, Kaushik Maiti, Chengbing Wang, Abdus Salam, Sang Young Chun, Won-Kyo Lee, Kyungjin Kim, Hyuk Bang Kwon, and Jae Young Seong

Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Gwangju, Republic of Korea (J.H.L., A.F.W., K.M., C.W., M.A.S., S.Y.C., H.B.K., J.Y.S.); Department of Physiology, Ulsan University College of Medicine, Seoul, Republic of Korea (H.C.); Department of Aquaculture, Division of Aqua Life Science, Yeosu National University, Jeollanam-Do, Republic of Korea (W.-K.L.); and School of Biological Sciences, Seoul National University, Seoul, Republic of Korea (K.K.)

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ABSTRACT

Mammalian type I and II gonadotropin-releasing hormone (GnRH) receptors (GnRHRs) show differential ligand preference for GnRH-I and GnRH-II, respectively. Using a variety of chimeric receptors based on green monkey GnRHR-2 (gmGnRHR-2), a representative type II GnRHR, and rat GnRHR, a representative type I GnRHR, this study elucidated specific domains responsible for this ligand selectivity. A chimeric gmGnRHR-2 with the extracellular loop 3 (EL3) and EL3-proximal transmembrane helix 7 (TMH7) of rat GnRHR showed a great increase in ligand sensitivity to GnRH-I but not to GnRH-II. Point-mutation studies indicate that four amino acids, Leu/Phe$^{7.38}$, Leu/Phe$^{7.43}$, Ala/Pro$^{7.46}$, and Pro/Cys$^{7.47}$ in TMH7 are critical for ligand selectivity as well as receptor conformation. Furthermore, a combinatory mutation (Pro$^{7.31}$-Pro$^{7.32}$-Ser$^{7.33}$ motif to Ser-Glu-Pro in EL3 and Leu$^{7.38}$, Leu$^{7.43}$, Ala$^{7.46}$, and Pro$^{7.47}$ to those of rat GnRHR) in gmGnRH-2 exhibited an approximately 500-fold increased sensitivity to GnRH-I, indicating that these residues are critical for discriminating GnRH-II from GnRH-I. [Trp^{7}]GnRH-I and [Trp^{8}]GnRH-I but not [His^{5}]GnRH-I exhibit a higher potency in activating wild-type gmGnRHR-2 than native GnRH-I, indicating that amino acids at positions 7 and 8 of GnRHs are more important than position 5 for differential recognition by type I and type II GnRHRs. As a whole, these data suggest a molecular coevolution of ligands and their receptors and facilitate the understanding of the molecular interaction between GnRHs and their cognate receptors.

Gonadotropin-releasing hormone receptor (GnRHR), a rhodopsin-like G protein-coupled receptor (GPCR), is one of the most extensively studied receptors because of its dual significance both for understanding reproductive biology and for the development of medical therapies (Sealfon et al., 1997). It is now well-established that most vertebrates, including human, have at least two forms of GnRH (White et al., 1998; Fernald and White, 1999). One form, GnRH-I (also called mammalian GnRH), is primarily synthesized in the hypothalamus, whereas the other form, GnRH-II (also called chicken GnRH-II), is widely expressed in the brain and peripheral tissues. Although GnRH-I is known to regulate the secretion and synthesis of gonadotropins in the pituitary, the exact function of GnRH-II is largely unknown. The receptor for GnRH-I was first isolated from mammalian pituitary cells (Kaiser et al., 1992; Reinhart et al., 1992; Tsutsumi et al., 1992) and called mammalian type I GnRHR. Receptors that have a high affinity for GnRH-II have been identified in nonmammalian and mammalian species (Tensen et al., 1997; Illing et al., 1999; Millar et al., 2001; Neill et al., 2001; Wang et al., 2001; Bogerd et al., 2002; Seong et al., 2003). Mammalian type II GnRHR is closer in structure to nonmammalian

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ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; gm, green monkey; GPCR, G protein-coupled receptor; EL3, extracellular loop 3; TMH, transmembrane helix; IP, inositol phosphate; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; r, rat; bf, bullfrog; r6TM, rat gonadotropin-releasing hormone receptor with the entire transmembrane helix 7 of green monkey gonadotropin-releasing hormone receptor-2.

GnRHRs than mammalian type I GnRHR. Mammalian type II GnRHR, like nonmammalian GnRHRs, contains the intracellular C-terminal tail, which is functionally important for desensitization and internalization (Heding et al., 1998; Willars et al., 1999), whereas mammalian type I GnRHR does not have a C-terminal tail. Mammalian type II and nonmammalian GnRHRs have $Asp^{2.50}$ and $Asp^{7.49}$ in the transmembrane helices (TMHs) 2 and 7, respectively, whereas mammalian type I GnRHRs contain $Asp^{2.50}$ and $Asn^{7.49}$, which are known to be important for receptor conformation and signal transduction (Blomenröhr et al., 1997; Mitchell et al., 1998).

Mammalian type II GnRHR has a higher affinity for GnRH-II than for GnRH-I, whereas the opposite is true for mammalian type I GnRHR. However, the factors that determine such differential ligand selectivity are poorly understood. Mutagenesis studies combined with computational modeling have identified a number of residues that are involved in ligand binding (Davidson et al., 1996; Flanagan et al., 2000; Hoffmann et al., 2000; Hövelmann et al., 2002). GnRH-II differs from GnRH-I by three amino acids at positions 5, 7, and 8; thus, searching for residues that may interact with them would help us to understand the mechanism underlying differential ligand selectivity. It has been proposed that Tyr^{5} and Leu^{7} of GnRH-I interact with $Tyr^{6.58}$ and $Trp^{2.64}$ of mammalian type I GnRHR (Hövelmann et al., 2002). However, because $Tyr^{6.58}$ and $Trp^{2.64}$ are also conserved in mammalian type II GnRHR, these residues alone cannot account for differential ligand selectivity. An acidic amino acid, $Glu/Asp^{7.32}$ in EL3 of mammalian type I GnRHR is known to confer ligand specificity for GnRH-I by an electrostatic interaction with Arg^{8} of GnRH-I (Flanagan et al., 1994; Fromme et al., 2001). However, this is not fully explanatory, because some nonmammalian GnRHRs have an acidic amino acid (e.g., $Glu^{7.32}$ for bfGnRHR-2 and $Asp^{7.32}$ for catfish GnRHR) at this homologous position, yet these receptors respond better to GnRH-II than to GnRH-I (Wang et al., 2001). We have demonstrated that the positions of Ser and Pro flanking $Glu/Asp^{7.32}$ are critical determinants for ligand selectivity (Wang et al., 2004). Replacement of the Ser-Glu-Pro (SEP) motif by Pro-Glu-Ser (PES) in mammalian type I GnRHR induced an increased sensitivity to GnRH-II but the opposite to GnRH-I. Moreover, mutation of a Ser-Gln-Ser (SQS) motif to SEP in bullfrog type I GnRHR (bfGnRHR-1) showed an increased sensitivity to GnRH-II but a decreased sensitivity to GnRH-I (Wang et al., 2004). However, this study found no reverse-ligand selectivity when the Pro-Glu-Tyr (PEY) motif in bfGnRHR-2 was replaced by SEP, suggesting the involvement of other residues in ligand selectivity.

Sequence alignments showed that the EL3-proximal TMH7 of mammalian type II GnRHR has a high degree of sequence identity with that of nonmammalian GnRHRs but not with that of mammalian type I GnRHR. In the present study, using rat GnRHR and gmGnRHR-2 as models for representative mammalian type I and type II GnRHRs, respectively, we addressed whether EL3 and/or EL3-proximal TMH7 determine differential ligand selectivity. Domain swapping and site-directed mutagenesis studies suggest that the Pro-Pro-Ser (PPS) motif in EL3 and $Leu^{7.38}$, $Leu^{7.43}$, $Ala^{7.46}$, and $Pro^{7.47}$ in TMH7 of gmGnRHR-2 are critical for discriminating GnRH-II from GnRH-I.

Materials and Methods

Materials. GnRH-I (pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-$GlyNH_2$), GnRH-II ([His^5, Trp^7, Tyr^8]GnRH-I), [His^5]GnRH-I, [Trp^7]GnRH-I, [Trp^8]GnRH-I, and [Trp^7, Leu^8]GnRH-I were synthesized by AnyGen (Gwangju, Korea). The c-fos-luc vector containing approximately −711 to +45 sequence of the human c-fos promoter constructed in the pFLASH vector was a kind gift from Dr. R. Prywes (Columbia University, New York, NY). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA). All oligonucleotides were synthesized from GenoTech (Daejon, Korea). GH_3 cell lines stably expressing gmGnRHR-2 or rat GnRHR were established as described previously (Acharjee et al., 2002; Wang et al., 2003).

Amino Acid Residue Numbering Scheme. Amino acid residues are numbered according to their positions in gmGnRHR-2. To facilitate the comparison among different GnRHRs, the standard numbering system proposed by Ballesteros and Weinstein (1995) was also used.

Construction of Wild-Type and Mutant GnRHRs. The cDNA of gmGnRHR-2 subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) at the KpnI and XbaI sites (Wang et al., 2003) was used as a template for creating domain-swapped or site-directed mutants. Domain-swapping and site-directed mutagenesis were performed by the polymerase chain reaction overlapping-extension method (Wang et al., 2003, 2004). To facilitate the construction of domain-swapped mutants, an exogenously introduced EcoRV site at the $Asn^{7.34}$ residue and an intrinsic BstXI site or two intrinsic BamHI sites were used. EL3 or EL3-proximal TMH7 of rat GnRHR was amplified using a specific set of primers flanked by the overlapping sequence of gmGnRHR-2 and the appropriate restriction endonuclease recognition site, producing rEL3S and rEL3L, respectively. The fragment from the N terminus to the EcoRV site at the $Asn^{7.34}$ residue of rEL3S was replaced by the corresponding fragment of rat GnRHR, generating the r6TM chimera. Likewise, the fragment from the N terminus to the BamHI site at the $Pro^{7.47}$ residue of gmGnRHR-2 was replaced by the corresponding part of rat GnRHR, producing the r6.5TM chimera. Mutated sequences were confirmed using the Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochemical Corporation, Cleveland, OH) according to the manufacturer's instructions.

Inositol Phosphate Production Assay. The inositol phosphate (IP) production assay was performed as described previously (Wang et al., 2003). GH_3 cells (1×10^5/well) expressing gmGnRHR-2 or rat GnRHR were seeded in 12-well plates, and the following day, cells were incubated in inositol-free DMEM (Invitrogen) containing 2% dialyzed fetal bovine serum and labeled with 1 μCi of [*myo*-^{3}H]inositol/well (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) for 18 h. Medium was then removed, and cells were washed with 0.5 ml of buffer A (140 mM NaCl, 20 mM HEPES, 4 mM KCl, 8 mM D-glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mg/ml fatty acid-free BSA). Cells were then preincubated with buffer A containing 10 mM LiCl for 15 min, followed by treatment with graded concentrations (0.01 nM to 10 μM) of GnRHs at 37°C for 45 min. The reaction was terminated by removing the incubation medium and adding 0.5 ml of ice-cold 10 mM formic acid. After 30 min at 4°C, the formic acid extracts were transferred into columns containing Dowex anion exchange resin. Total IPs were then eluted with 1 ml of 1 M ammonium formate/0.1 M formic acid, and their radioactivity was determined.

Luciferase Assay. Wild-type and mutant GnRHRs were transiently transfected into CV-1 cells, which were maintained at 37°C in DMEM with 10% heat-inactivated fetal bovine serum, 1 mM glutamate, 100 U of penicillin, and 100 μg/ml streptomycin. Cells were seeded in 24-well plates (1×10^5/well), and transfection was performed using the SuperFect transfection kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions with a minor modification. For each transfection, 100 ng of each receptor cDNA, 200 ng of c-fos-luc vector, and 200 ng of internal control plasmid pCMVβ-Gal were used. One day after transfection, cells were serum-starved for

24 h and then challenged with GnRH for 6 h (Oh et al., 2003). Cells were harvested, and luciferase activity in the cell extract was determined according to standard methods in a Lumat LB9501 (Berthold Technologies, Bad Wildbad, Germany). The luciferase activities were normalized using β-galactosidase values. Transfection experiments were performed in duplicate and were repeated three to five times.

Binding Assay. GnRH-II was radioiodinated using the chloramine-T method and purified by chromatography on a Sephadex G-25 (Sigma-Aldrich, St. Louis, MO) column in 0.01 M acetic acid and 0.1% BSA. HeLa cells were transfected with wild-type, individual mutant construct, or pcDNA3 (300 ng of DNA per well in 12-well plates) with Effectene (QIAGEN) according to the manufacturer's instructions. Thirty-six hours after transfection, intact cells were washed and incubated with binding buffer (DMEM supplemented with 0.1% BSA, pH 7.4) containing 250,000 cpm of ^{125}I-GnRH-II (0.5 ml final volume) at 20°C for 1 h to achieve equilibrium. Specific binding was calculated by subtracting nonspecific binding (the presence of 10 μM unlabeled GnRH-II) from total binding. For the displacement binding assay, ^{125}I-GnRH-II was incubated in the presence of graded concentrations of unlabeled GnRH-I or GnRH-II.

Molecular Modeling. gmGnRHR-2 was built by MODELLER 6 version 2 (Sali and Blundell, 1993) on the basis of the crystal structure of bovine rhodopsin (Okada et al., 2002) as a template. GnRH-I or GnRH-II was docked onto putative binding sites of gmGnRHR-2 manually using Visual Molecular Dynamics (Humphrey et al., 1996): PyroGlu^{1} with Asn$^{5.39}$, His^{2} with Asp$^{2.61}$, Trp^{3} with Asn$^{6.48}$, Tyr^{5} with Tyr$^{6.58}$, and Gly^{10} with Asp$^{2.61}$ and Asn$^{2.65}$. The models for gmGnRHR-2/GnRH-II, mutant gmGnRHR-2/GnRH-I, and mutant gmGnRHR-2/GnRH-II were built by mutating corresponding residues in the gmGnRHR-2/GnRH-I model and underwent energy minimization and molecular dynamics annealing simulations in the MODELLER. The final models showing good geometry were confirmed by PROCHECK (Laskowski et al., 1993). The contacts between ligands and receptors were analyzed using Ligplot (Wallace et al., 1995). Figures of the models were drawn using Visual Molecular Dynamics (Humphrey et al., 1996).

Data Analysis. Analyses were performed using nonlinear regression, and the data were expressed as sigmoid dose-response curves. GnRH concentrations inducing half-maximal stimulation (EC_{50}), half-maximal inhibition (IC_{50}), and maximal fold increases (E_{max}) were calculated using GraphPad Prism 3 software (GraphPad Software Inc., San Diego, CA). All data are presented as means ± S.E.M. The data were analyzed by one-way analysis of variance followed by the Bonferroni test. A p value <0.05 was considered statistically significant.

Results

Differential Ligand Selectivity of Mammalian and Nonmammalian GnRHRs. The ligand selectivities of rat GnRHR and gmGnRHR-2 were examined using two different methods: IP production, and c-fos promoter-driven luciferase (c-fos-luc) assays. For the IP assay, GH_3 cells stably expressing rat GnRHR or gmGnRHR-2 were used (Wang et al., 2003; Maiti et al., 2003), and for c-fos-luc assay, CV-1 cells transiently expressing rat GnRHR or gmGnRHR-2 were used. As for rat GnRHR, GnRH-I showed a lower EC_{50} value than did GnRH-II, indicating that rat GnRHR has a higher sensitivity to GnRH-I than to GnRH-II. However, gmGnRHR-2 responded better to GnRH-II than did GnRH-I in both assay systems (Fig. 1 and Table 1). Regarding GnRHR, GnRH-I had a 7.4- and 5.9-fold higher potency than GnRH-II in IP and c-fos-luc assay systems, respectively. For gmGnRHR-2, GnRH-II was 204-fold (IP assay) and 239-fold (c-fos-luc assay) more potent than GnRH-I (Table 1). Because c-fos-luc was more sensitive than the IP assay system, we used the c-fos-luc system in ensuing experiments.

EL3 and EL3-Proximal TMH7 Are Involved in Differential Ligand Selectivity. Sequence alignment showed that EL3-proximal TMH7 of mammalian type II GnRHR has a high degree of sequence identity with that of nonmammalian GnRHRs but not mammalian type I GnRHR. Furthermore, it was suggested that the proximal region of TMH7 of GnRHR affects the conformation of EL3 (Petry et al., 2002). We therefore presumed that both EL3 and the EL3-proximal TMH7 may be involved in differential ligand selectivity. To address this possibility, EL3 alone or together with EL3-proximal TMH7 of gmGnRHR-2 was swapped with that of rat GnRHR, designated rEL3S or rEL3L, respectively. Swapping EL3 alone did not induce a significant change in sensitivity for either GnRH-I or GnRH-II such that chimeric rEL3S, like

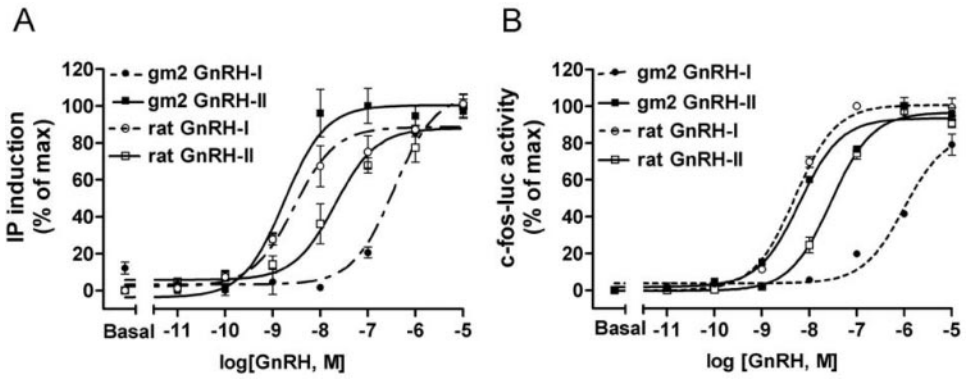


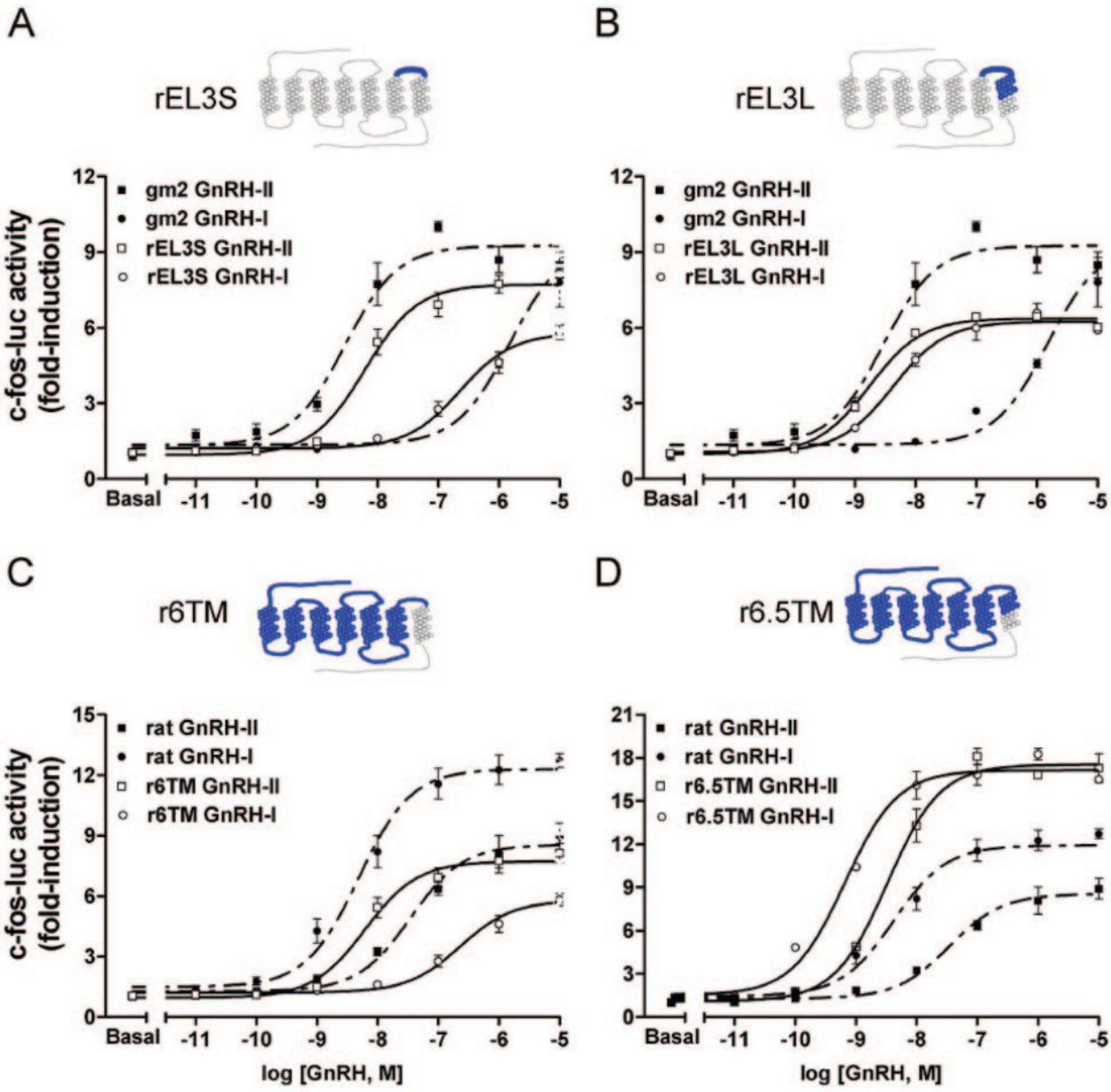
Fig. 1. Ligand selectivity of rat GnRHR or gmGnRHR-2. A, IP assays were performed using GH_3 cells which stably express rat GnRHR (rat) or gmGnRHR-2 (gm2). Cells were treated with increasing concentrations of GnRH-I or GnRH-II for 30 min. B, c-fos-luc activity was examined in CV-1 cells that were cotransfected with 200 ng of c-fos-luc reporter vector plus rat GnRHR or gmGnRHR-2. Twenty-four hours after transfection, cells were serum-starved for 18 h and then treated for 6 h with GnRH-I or GnRH-II. Cell lysates were used for luciferase assays. Broken lines are dose-responses of rat (○) and gm2 (●) to GnRH-I, and solid lines are dose-responses of rat (□) and gm2 (■) to GnRH-II.

TABLE 1

The differential ligand selectivity between rat GnRHR (rat) and gmGnRHR-2 (gm2)

Values given form IP and c-fos-luc are log(EC_{50}, M). Data represent the mean ± S.E.M. from three independent experiments. Numbers in parentheses represent fold difference in sensitivity between GnRH-I and GnRH-II.

	IP		c-fos-luc	
	GnRH-I	GnRH-II	GnRH-I	GnRH-II
gm2	−6.45 ± 0.11	−8.76 ± 0.14 (204)	−5.83 ± 0.10	−8.21 ± 0.09 (239)
Rat	−8.55 ± 0.14	−7.68 ± 0.15 (7.4)	−8.31 ± 0.06	−7.54 ± 0.07 (5.9)



E

			EL3	TMH7		
rGnRHR	288	...WYWF	DPEMLNRV**SEP**VN	HF**F**FLFA**F**LN**PC**	FDPL...	320
gmGnRHR-2	286	...WYWF	SPTMLTEV**PPS**LS	HI**L**FLFG**L**LN**AP**	LDPL...	318
rEL3L	286	...WYWF	DPEMLNRV**SEP**VN	HF**F**FLFA**F**LN**PC**	LDPL...	318
rEL3S	286	...WYWF	DPEMLNRV**SEP**VN	HI**L**FLFG**L**LN**AP**	LDPL...	318
r6TM	288	...WYWF	DPEMLNRV**SEP**VN	HI**L**FLFG**L**LN**AP**	LDPL...	320
r6.5TM	288	...WYWF	DPEMLNRV**SEP**VN	HF**F**FLFA**F**LN**PC**	LDPL...	320
			7.32	7.38 7.43 7.46 7.47		

Fig. 2. Ligand selectivity of chimeric GnRHRs. A–D, chimeric receptors rEL3S (A), rEL3L (B), r6TM (C), and r6.5TM (D) were transfected into CV-1 cells, and c-fos-luc activity was measured. Broken lines are dose-response c-fos-luc activity of wild-type gmGnRHR-2 (gm2) or rat GnRHR (rat), and solid lines are that of chimeric receptors. Thin gray lines in the receptor diagram represent the portion of gmGnRHR-2, whereas thick blue lines are the region from rat GnRHR. E, the amino acid sequence alignment of EL3 and TMH7 among wild-type and chimeric receptors. The amino acid numbers are shown beside the sequences. Insertions in rEL3S and rEL3L are underlined. Amino acids that are further characterized in ensuing experiments are shown in boldface type and are indicated by arrows with the position numbers.

wild-type gmGnRHR-2, showed a higher sensitivity to GnRH-II than to GnRH-I (Fig. 2A). It is interesting that rEL3L showed a great increase in sensitivity to GnRH-I but not to GnRH-II (Fig. 2B and Table 2), indicating that EL3-proximal TMH7 in gmGnRHR-2 is probably important for the discrimination between them. The functional importance of EL3-proximal TMH7 was further confirmed by additional chimeric receptors that have N termini to EL3 or to EL3-proxmial TMH7 of rat GnRHR, denoted r6TM or r6.5TM, respectively. The chimeric receptor r6TM, which has the EL3-proximal TMH7 sequence of gmGnRHR-2, has a high sensitivity to GnRH-II and a low sensitivity to GnRH-I, characteristics of a type II receptor (Fig. 2C). In contrast, r6.5TM containing the EL3-proximal TMH7 sequence of rat GnRHR has the ligand sensitivity, characteristic of a type I GnRHR (Fig. 2D), again confirming that EL3-proximal TMH7 in gmGnRHR-2 is critical for ligand selectivity.

Identification of rEL3L Amino Acids Involved in Ligand Selectivity. Because sequence alignment showed a six amino acid difference in EL3-proximal TMH7 between gmGnRHR-2 and rat GnRHR (Fig. 2E), we postulated that one of them may be responsible for ligand selectivity. Thus, six individual amino acids were reciprocally changed in the rEL3L chimeric receptor. Point mutation of $Phe^{7.37}$ to isoleucine, $Phe^{7.38}$ to leucine, or $Ala^{7.42}$ to glycine did not induce significant changes in ligand selectivity compared with rEL3L (Fig. 3, A and B, and Table 2). Point mutation of $Pro^{7.46}$ to alanine or $Cys^{7.47}$ to proline completely suppressed receptor function in response to either GnRH-I or GnRH-II (Fig. 3, C and D). Finally, the mutation of $Phe^{7.43}$ to leucine in rEL3L showed a significant decrease in sensitivity to GnRH-I (Fig. 3A).

It was shown previously that $Glu^{7.32}$ of mouse GnRHR is a critical residue conferring ligand specificity for Arg^{8} of GnRH-I (Flanagan et al., 1994). Furthermore, we recently demonstrated that the positions of serine and proline flanking $Glu^{7.32}$ are crucial for the ligand selectivity between mammalian and nonmammalian GnRHRs (Wang et al., 2004). Therefore, we sought to determine whether these amino acids are critical for the ligand selectivity in the chimeric receptor rEL3L. The $Glu^{7.32}$ or SEP motif in rEL3L was changed to glycine or PPS, respectively. A mutation of $Glu^{7.32}$ to glycine significantly decreased sensitivity to GnRH-I but not to GnRH-II (Fig. 3, C and D, and Table 2). Moreover, replacement of the SEP motif by PPS greatly increased sensitivity to GnRH-II, whereas it slightly decreased sensitivity to GnRH-I (Fig. 3, C and D, and Table 2). These results suggest that the SEP/PPS motif, together with amino acids in TMH7, is important in ligand sensitivity.

Identification of Amino Acids in Wild-Type gmGnRHR-2 That Confer Differential Ligand Selectivity. Because we observed that the amino acid residues $Phe^{7.43}$, $Pro^{7.46}$, and $Cys^{7.47}$ in rEL3L critically affected receptor activation and ligand selectivity, we further examined the function of these residues in wild-type gmGnRHR-2. Two mutants, $L^{7.43}F$ and $A^{7.46}P$, had no receptor activity (Fig. 4). The mutant $P^{7.47}C$ had essentially the same ligand selectivity as the wild-type gmGnRHR-2 (Fig. 4). Because we failed to observe reverse-ligand selectivity by a single mutation, we postulated that multiple amino acids are involved in ligand selectivity. To address this, double or triple mutants with different combinations of $Leu^{7.43}$, $Ala^{7.46}$, and $Pro^{7.47}$ were constructed. It is interesting that a double mutant, $A^{7.46}P/P^{7.47}C$, exhibited an improvement in ligand selectivity for

TABLE 2

Relative binding, E_{max}, and EC_{50} values of various GnRH receptors

Values represent the mean ± S.E.M. of three independent experiments performed in duplicate. Binding was expressed as a percentage of specific binding of gmGnRHR-2 (gm2).

Receptors	Relative Binding	GnRH-I		GnRH-II	
		E_{max}	EC_{50}	E_{max}	EC_{50}
	% gm2	*fold induction*	*log M*	*fold induction*	*log M*
gm2	100 ± 1.2	9.33 ± 0.35	−6.00 ± 0.10	13.82 ± 0.35	−8.54 ± 0.13
rat	98.8 ± 1.8	12.49 ± 0.26	−8.31 ± 0.06*	8.78 ± 0.29	−7.54 ± 0.07*
r6TM	89.5 ± 1.1	9.59 ± 0.33	−7.16 ± 0.12*	5.45 ± 0.20	−7.95 ± 0.12
r6.5TM	168.1 ± 4.5	17.14 ± 0.38	−9.18 ± 0.08*	17.53 ± 0.35	−8.48 ± 0.07
rEL3S	21.8 ± 0.1	5.79 ± 0.18	−6.64 ± 0.10	7.72 ± 0.21	−8.21 ± 0.09
rEL3L	110.1 ± 5.4	6.24 ± 0.14	−8.38 ± 0.09*	6.37 ± 0.08	−8.76 ± 0.05
rEL3L/$F^{7.37}I$	110.3 ± 9.4	6.83 ± 0.31	−8.25 ± 0.16*	10.77 ± 0.53	−8.24 ± 0.18
rEL3L/$F^{7.38}L$	138.7 ± 1.1	7.63 ± 0.35	−7.98 ± 0.18*	10.56 ± 0.29	−8.34 ± 0.10
rEL3L/$A^{7.42}G$	49.7 ± 3.4	6.31 ± 0.22	−8.25 ± 0.09*	5.43 ± 0.12	−8.79 ± 0.10
rEL3L/$F^{7.43}L$	52.7 ± 0.1	3.89 ± 0.27	−7.81 ± 0.20*	4.61 ± 0.19	−8.63 ± 0.18
rEL3L/$P^{7.46}A$	3.8 ± 0.5	N.D.			
rEL3L/$C^{7.47}P$	1.5 ± 1.0	N.D.			
rEL3L/$E^{7.32}Q$	70.7 ± 0.2	3.53 ± 0.14	−7.28 ± 0.15*	4.61 ± 0.14	−8.47 ± 0.14
rEL3L/PPS	332.2 ± 7.3	12.50 ± 0.27	−7.61 ± 0. 07*	16.78 ± 0.35	−9.36 ± 0.09*
$L^{7.43}F$	1.7 ± 1.2	N.D.			
$A^{7.46}P$	3.8 ± 0.9	N.D.			
$P^{7.47}C$	72.1 ± 2.1	6.46 ± 0.26	−5.93 ± 0.08	12.29 ± 0.57	−8.29 ± 0.17
$L^{7.43}F/A^{7.46}P$	3.2 ± 2.8	N.D.			
$L^{7.43}F/P^{7.47}C$	16.8 ± 1.2	3.57 ± 0.07	−7.03 ± 0.07*	4.94 ± 0.15	−8.79 ± 0.17
$A^{7.46}P/P^{7.47}C$	303.3 ± 1.4	10.53 ± 0.61	−7.17 ± 0.17*	11.00 ± 0.29	−9.65 ± 0.12*
$L^{7.43}F/A^{7.46}P/P^{7.47}C$	178.6 ± 8.6	5.93 ± 0.20	−8.21 ± 0.13*	7.06 ± 0.20	−9.82 ± 0.13*
SEP/$I^{7.43}F/A^{7.46}P/P^{7.47}C$	11.1 ± 0.5	2.92 ± 0.08	−7.87 ± 0.12*	2.53 ± 0.10	−9.69 ± 0.26*
rEL3S/$L^{7.43}F/A^{7.46}P/P^{7.47}C$	54.6 ± 2.1	4.68 ± 0.13	−8.76 ± 0.15*	6.14 ± 0.11	−9.59 ± 0.10*
SEP/$I^{7.37}F/L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$	51.2 ± 1.3	3.99 ± 0.14	−8.59 ± 0.17*	4.76 ± 0.17	−9.32 ± 0.17*
SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$	62.1 ± 1.7	5.27 ± 0.13	−8.79 ± 0.11*	5.79 ± 0.14	−9.43 ± 0.11*

N.D., not determined.

* $P < 0.05$ versus wild-type gm2.

both GnRH-I and GnRH-II (Fig. 5, A and B). The double mutant $L^{7.43}F/P^{7.47}C$ showed a decrease in receptor efficacy in both GnRH-I and GnRH-II but showed a slight increase in sensitivity for GnRH-I (Fig. 5, A and B). The double mutant $L^{7.43}F/A^{7.46}P$ did not respond to GnRH stimulation (Fig. 5, A and B). A triple mutant, $L^{7.43}F/A^{7.46}P/P^{7.47}C$, exhibited a large increase in ligand sensitivity for both GnRH-I and GnRH-II. Compared with wild-type gmGnRHR-2, the $L^{7.43}F/$

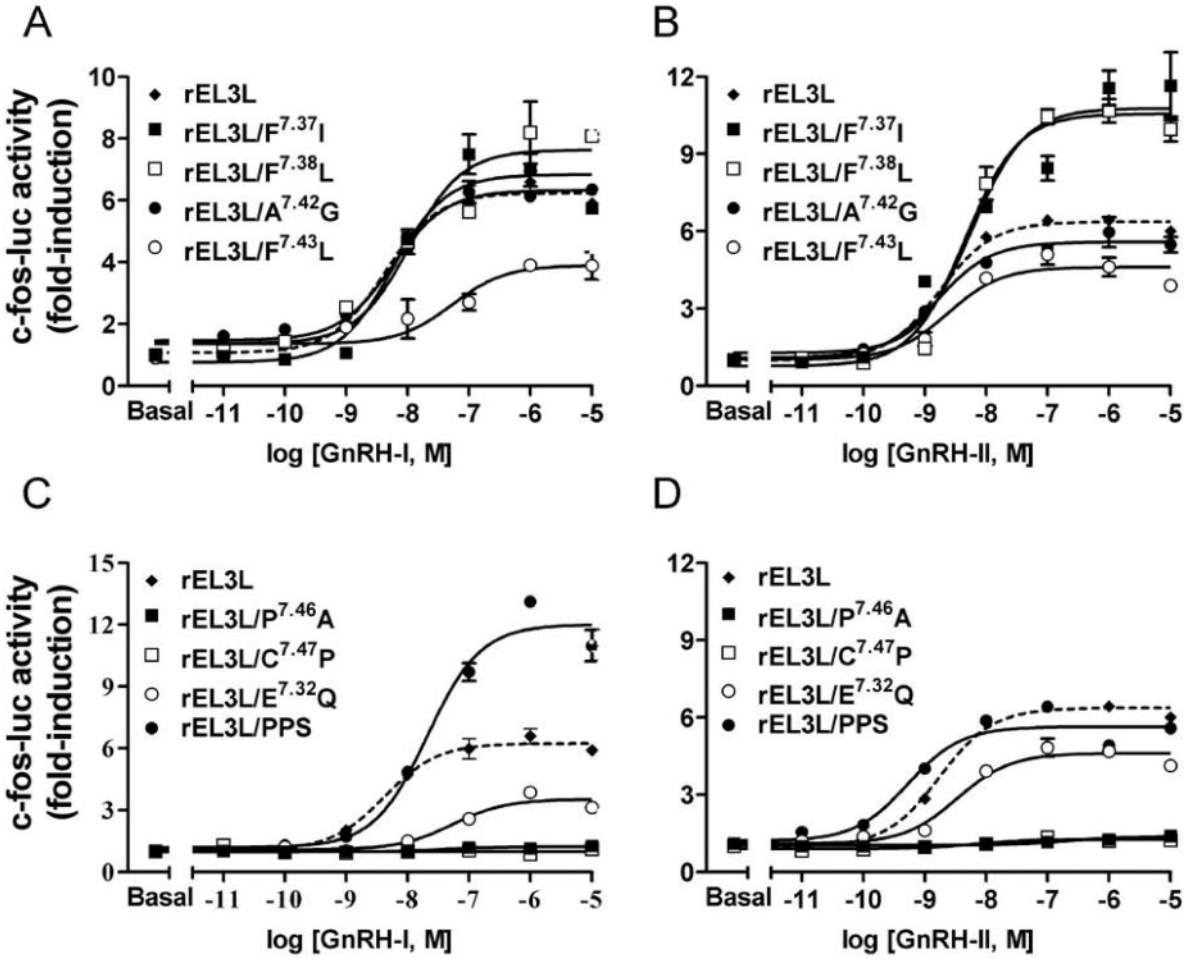


Fig. 3. Point mutation of the rEL3L mutant. CV-1 cells were transfected with 200 ng of c-fos-luc reporter plasmid plus rEL3L or rEL3L with point mutations. A and B, c-fos-luc activity in cells expressing $rEL3L/F^{7.37}I$, $rEL3L/F^{7.38}L$, $rEL3L/A^{7.42}G$, $rEL3L/F^{7.43}L$ in response to GnRH-I (A) and GnRH-II (B). C and D, c-fos-luc activity in cells expressing $rEL3L/P^{7.46}A$, $rEL3L/C^{7.47}P$, $rEL3L/E^{7.32}Q$, and rEL3L/PPS in response to GnRH-I (C) and GnRH-II (D). Broken and solid lines are data obtained from rEL3L and the mutant receptors, respectively.

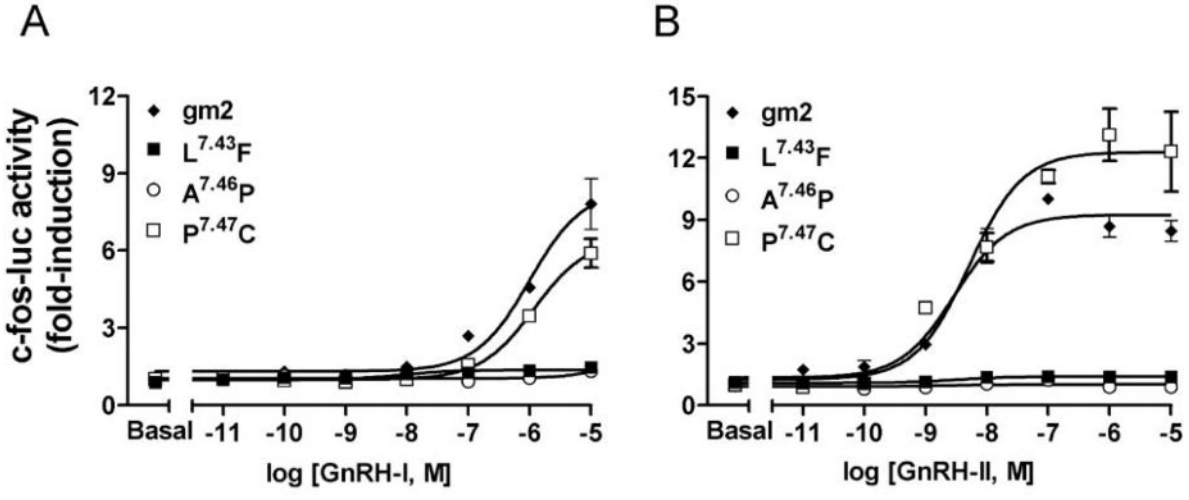


Fig. 4. Point mutations of wild-type gmGnRHR-2. CV-1 cells were transiently transfected with gmGnRHR-2 (gm2) and point-mutated receptors $L^{7.43}F$, $A^{7.46}P$, and $P^{7.47}C$. Cells were treated with different concentrations of GnRH-I (A) or GnRH-II (B) for 6 h.

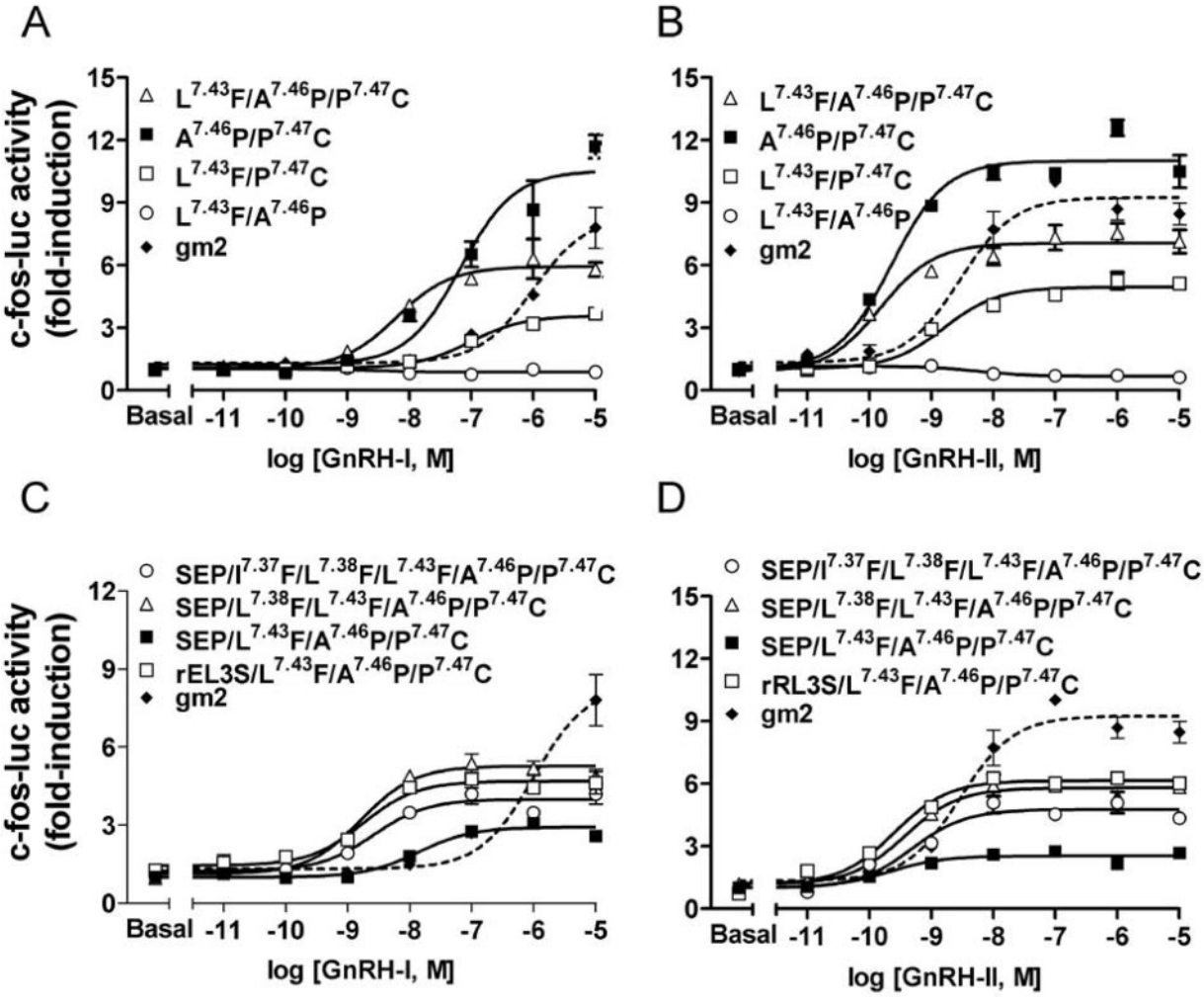


Fig. 5. Multiple mutations in EL3 and TMH7 of gmGnRHR-2. CV-1 cells were transfected with wild-type gmGnRHR-2 or each mutant. A and B, cells expressing $A^{7.46}P/P^{7.47}C$, $L^{7.43}F/P^{7.47}C$, $L^{7.43}F/A^{7.46}P$, and $L^{7.43}F/A^{7.46}P/P^{7.47}C$ were treated with different concentrations of GnRH-I (A) or GnRH-II (B) for 6 h. C and D, cells expressing combinatory mutants $SEP/L^{7.43}F/A^{7.46}P/P^{7.47}C$, $rEL3S/L^{7.43}F/A^{7.46}P/P^{7.47}C$, $SEP/I^{7.37}F/L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$, and $SEP/L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ were treated with GnRH-I (C) or GnRH-II (D). Broken lines are data from wild-type receptor (gm2), and solid lines are from mutant receptors, respectively.

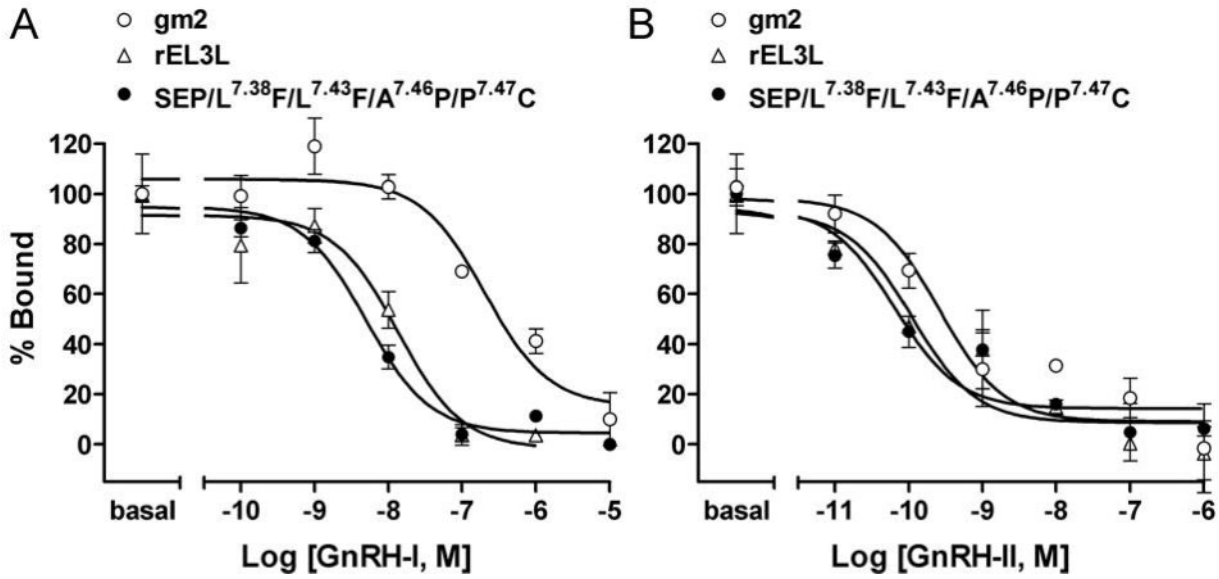


Fig. 6. Competition binding assays for the wild-type and mutant receptors. [^{125}I]GnRH-II was applied to HeLa cells expressing the wild-type (gm2), rEL3L, and $SEP/L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutant receptors in the presence of graded concentrations of unlabeled GnRH-I (A) or GnRH-II (B).

$A^{7.46}P/P^{7.47}C$ mutant showed a ~200-fold increased sensitivity to GnRH-I and a ~20-fold increased sensitivity to GnRH-II (Fig. 5, A and B).

Because the PPS/SEP motif in EL3 and $Leu^{7.43}$, $Ala^{7.46}$, and $Pro^{7.47}$ residues in TMH7 affect ligand selectivity, we examined a combinatory effect of these two motifs. The gmGnRHR-2 with SEP/$L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutation revealed a slight decrease in sensitivity for both GnRH-I and GnRH-II (Fig. 5, C and D). This mutant also showed a decrease in E_{max} values for both GnRHs compared with the $L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutant, which may be caused by low receptor expression (Table 2). It is interesting that additional mutations rEL3S/$L^{7.43}F/A^{7.46}P/P^{7.47}C$ (Fig. 7B), SEP/$I^{7.37}F/L^{.38}F/L^{.43}F/A^{7.46}P/P^{7.47}C$ (Fig. 5, C and D), or SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ (Fig. 5, C and D) increased sensitivity to GnRH-I and decreased sensitivity to GnRH-II (Table 2).

Ligand Binding Affinities. Ligand affinities of wild-type, rEL3L, and SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutants were determined using a competition binding assay. For the binding assay, HeLa cells were used because when transfected with the receptors, they have a much higher binding capacity than CV-1 cells. It should be noted that HeLa cells, in the c-fos-luc assay system, produce EC_{50} values similar to those of CV-1 cells when we applied the same receptor and ligand (data not shown). HeLa cells, however, have a high basal c-fos-luc activity; therefore, their fold increases are usually much lower than those in CV-1 cells (Oh et al., 2003). [^{125}I]GnRH-II (250,000 cpm) was applied to HeLa cells expressing wild-type and mutant receptors in the presence of graded concentrations of unlabeled GnRH-I or GnRH-II. Log IC_{50} values for GnRH-I in cells expressing rEL3L (-7.87 ± 0.22) and SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ (-8.30 ± 0.11) were significantly lower compared with those in cells expressing the wild-type receptor (-6.69 ± 0.21) (Fig. 6A), indicating an increased affinity for GnRH-I in the mutant receptors. Log IC_{50} values for GnRH-II in cells expressing the wild-type, rEL3L, and SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutants were -9.58 ± 0.27, -10.19 ± 0.23, and -9.96 ± 0.27, respectively (Fig. 6B), showing that ligand affinities for GnRH-II in mutant receptors do not change as drastically as those for GnRH-I.

Relative ligand binding of mutant constructs was determined using [^{125}I]GnRH-II in the absence or presence of unlabeled GnRH-II (10 μM). For wild-type gmGnRHR-2, total and nonspecific binding were $2.3 \pm 0.2\%$ (5826 ± 50 cpm) and $0.60 \pm 0.2\%$ (1567 ± 30 cpm), respectively. Total binding for other mutant receptors ranged from 1.16 to 6.83%, whereas nonspecific bindings for other receptors were the same as that for the wild-type receptor. Mutants that did not respond to GnRHs (rEL3L/$P^{313}A$, rEL3L/$C^{314}P$, $L^{7.43}F$, and $A^{7.46}P$) were unable to bind radioiodinated GnRH-II. rEL3S, $L^{7.43}F/P^{7.47}C$, and SEP/$L^{7.43}F/A^{7.46}P/P^{7.47}C$ showed relatively low binding; rEL3L/PPS and $A^{7.46}P/P^{7.47}C$ had higher binding than gmGnRHR-2. Other mutants exhibited 49.7 to 178.6% binding compared with wild-type gmGnRHR-2 (Table 2).

Ligand Sensitivity for Chimeric GnRHs. Natural and chimeric GnRHs, in which amino acids at positions 5, 7, and 8 were substituted, were used to examine ligand sensitivity of gmGnRHR-2, rEL3L, and SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutants. For wild-type gmGnRHR-2, all chimeric GnRHs ([His^5]GnRH-I, [Trp^7]GnRH-I, [Trp^8]GnRH-I, and [Trp^7, Leu^8]GnRH-I) exhibited a higher potency than GnRH-I (Table 3). In particular, substitution of the amino acid residues at positions 7 and 8 of GnRH-I greatly increased potency to activate gmGnRHR-2. It should be noted that chimeric ligands [His^5]GnRH-I and [Trp^7]GnRH-I, which, like GnRH-I, retain Arg^8, showed a 100- to 200-fold increased potency for either rEL3L or SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ that have enhanced sensitivity to GnRH-I (Fig. 7 and Table 3). [Trp^8]GnRH-I revealed a 20- to 50-fold increased sensitivity for rEL3L and SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ compared with that for wild-type gmGnRHR-2. It is interesting that rEL3L and SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ had similar sensitivity to chimeric GnRHs. (Fig. 7 and Table 3). This result supports the idea that positions 7 and 8 in GnRH are important for conferring its specificity.

Molecular Modeling. To support our biochemical data, we constructed models to simulate the interaction of GnRHs with wild-type gmGnRHR-2 and SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutant (Fig. 8). Overall, the models agree well with previous reports (Hövelmann et al., 2002; Wang et al., 2004): two cysteine residues (Cys^{113} and Cys^{188}) of the receptors are close because they are involved in a disulfide bond; $pGlu^1$ of the ligands formed hydrogen bonds with $Asn^{5.39}$ of the receptors; Trp^3 of the ligands was located in the aromatic cage formed by $Trp^{6.48}$, $Phe^{5.43}$, and $Tyr^{6.52}$ of the receptors; Arg^8 of GnRH-I formed an ionic interaction with $Glu^{7.32}$ of the SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutant.

GnRH-II and GnRH-I differ by three residues: His/Tyr^5, Trp/Leu^7, and Tyr/Arg^8. Trp^7 of GnRH-II made a hydrophobic contact with $Pro^{7.32}$ of gmGnRHR-2. Tyr^8 had an interaction with $Pro^{7.32}$ and $His^{7.36}$ (Fig. 8A). In addition, Trp^7 formed a hydrogen bond with the carbonyl oxygen of $Val^{7.30}$, and Tyr^8 did so with the backbone of $Ser^{7.33}$. However, in the GnRH-I/gmGnRHR-2 complex, Leu^7 formed a hydrophobic contact with $Tyr^{6.58}$. Arg^8 moved to EL2 and interacted with $Val^{4.67}$ via a hydrophobic interaction (Fig. 8B). On the other hand, Trp^7 of GnRH-II formed hydrophobic contacts with $Glu^{7.32}$ and $His^{7.36}$ of the SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$

TABLE 3
E_{max} and EC_{50} values for various chimeric GnRHs
Data represent the mean ± S.E.M. of three independent experiments performed in duplicate.

Chimeric GnRH	gm2		rEL3L		SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$	
	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}
	fold induction	*log M*	*fold induction*	*log M*	*fold induction*	*log M*
GnRH-I	9.33 ± 0.35	−6.00 ± 0.10	6.24 ± 0.14	−8.38 ± 0.09	5.27 ± 0.13	−8.79 ± 0.11
[His^5]GnRH-I	8.66 ± 1.11	−6.38 ± 0.34	6.82 ± 0.39	−8.51 ± 0.22	4.78 ± 0.21	−8.53 ± 0.18
[Trp^7]GnRH-I	10.73 ± 0.05	−7.15 ± 0.05*	3.38 ± 0.14	−9.42 ± 0.26*	3.00 ± 0.19	−8.90 ± 0.34
[Trp^8]GnRH-I	9.48 ± 0.11	−6.74 ± 0.03*	4.80 ± 0.17	−8.16 ± 0.15	3.42 ± 0.20	−8.30 ± 0.24
[Trp^7, Leu^8]GnRH-I	12.11 ± 0.42	−7.47 ± 0.11*	5.68 ± 0.38	−8.43 ± 0.27	5.94 ± 0.43	−7.59 ± 0.23*

* $P < 0.05$ versus GnRH-I

mutant, and Tyr[8] made a hydrogen bond with the backbone nitrogen of Glu[7.32] (Fig. 8C). In the complex of GnRH-I with the SEP/L[7.38]F/L[7.43]F/A[7.46]P/P[7.47]C mutant, Leu[7] formed hydrophobic contact with Tyr[6.58], and Arg[8] had an ionic interaction with Glu[7.32] (Fig. 8D). Mutation of the four amino acids at TMH7 altered intramolecular interactions. For instance, the hydrogen bond between Leu[7.43] and Ala[7.46] of gmGnRHR-2 was suppressed in the SEP/L[7.38]F/L[7.43]F/A[7.46]P/P[7.47]C mutant. In addition, novel hydrophobic contacts of Phe[7.38] with Leu[6.54], Phe[7.43] with Asp[2.61], and Pro[7.46] with Leu[7.44] were formed in the mutant receptor. It is also notable that the various intramolecular interactions were highly dependent on ligand type. Hydrophobic contacts of Phe[7.43] with Leu[1.42] and Val[2.57], Cys[7.47] with Gly[1.49], and hydrogen bonds between Cys[7.47] and Ser[1.45] and Asn[1.50] were present in the mutant receptor/GnRH-I complex, but these interactions were absent in the mutant receptor/GnRH-II complex. In contrast, the hydrophobic contacts of Phe[7.38] with Leu[6.53] and Pro[7.46] with Val[2.53] were present in the mutant receptor/GnRH-II complex.

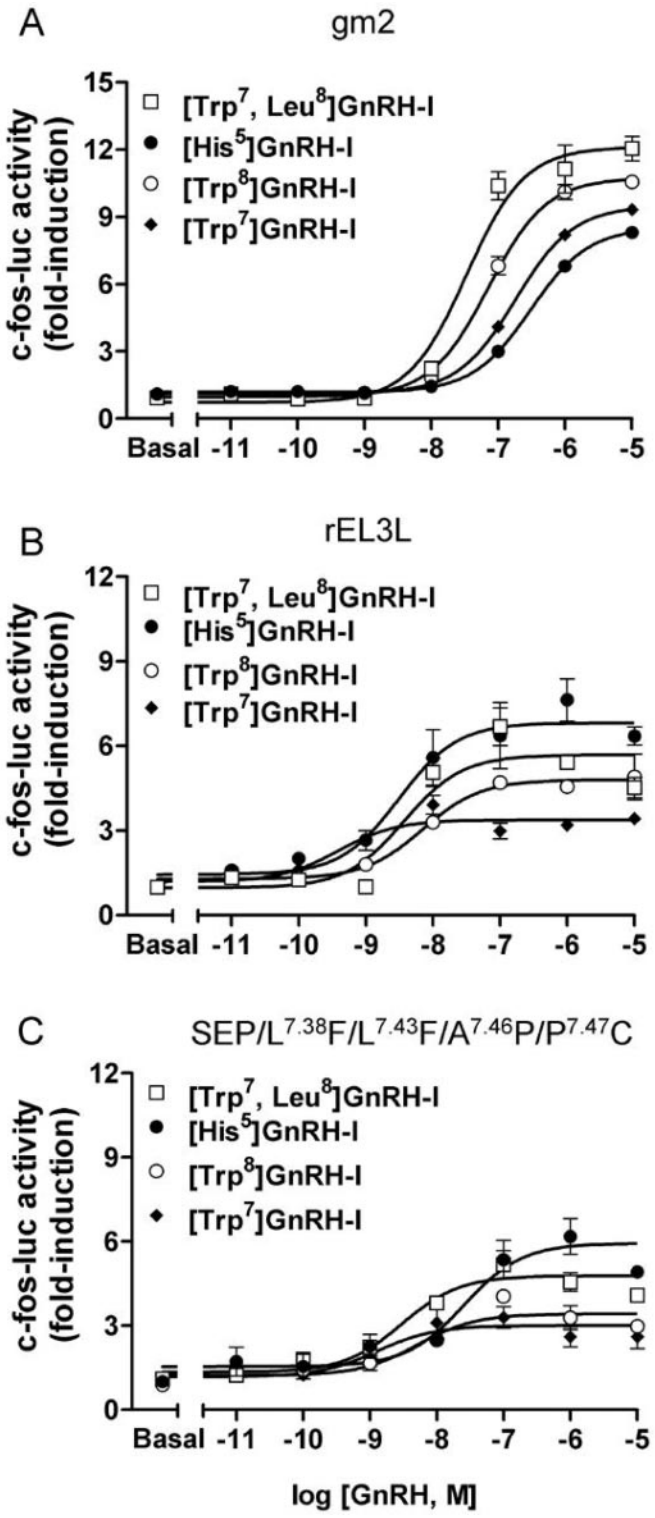


Fig. 7. Ligand selectivity of the chimeric receptors to various chimeric GnRHs. CV-1 cells were transfected with wild-type (gm2) (A), rEL3L (B), or SEP/L[7.38]F/L[7.43]F/A[7.46]P/P[7.47]C (C). Cells were treated with different concentrations of wild-type and chimeric GnRHs for 6 h.

Discussion

The present study demonstrates that replacement of EL3 and EL3-proximal TMH7 of gmGnRHR-2 with those of rat GnRHR greatly improves ligand sensitivity to GnRH-I but not to GnRH-II. Site-directed mutations on gmGnRHR-2 and back mutations on the domain-swapped receptor show that the PPS motif in EL3 and Leu[7.38], Leu[7.43], Ala[7.46], and Pro[7.47] in TMH7 of gmGnRHR-2 and the corresponding residues of rat GnRHR are responsible for differential ligand sensitivity to GnRH-I and GnRH-II.

It was suggested that not only Glu/Asp[7.32] but also the positions of Ser and Pro flanking Glu/Asp[7.32] in EL3 of mammalian type I GnRHR determine high selectivity for GnRH-I (Fromme et al., 2004; Wang et al., 2004). These findings indicate that a local conformation of EL3 is critical for differential ligand selectivity among nonmammalian and mammalian types I and II GnRHRs. However, replacement of EL3 from gmGnRHR-2 alone does not affect ligand selectivity to GnRH-I and GnRH-II. Likewise, substitution of SEP for the PEY motif of bfGnRHR-2 does not alter ligand sensitivity to GnRH-I and GnRH-II (Wang et al., 2004). These observations suggest that other amino acid residues/motifs are involved in the selectivity of GnRH. Our study strongly suggests that in mammalian type II GnRHR, EL3-proximal TMH7 in addition to EL3 participates in differential ligand selectivity. The importance of EL3-proximal TMH7 in ligand sensitivity is supported by the observation that rat GnRHR with the entire TMH7 of gmGnRHR-2 (r6TM) exhibits a significant decrease in sensitivity for GnRH-I. Mutations of Pro[7.47] to cysteine combined with the mutation of Leu[7.43] to phenylalanine and/or Ala[7.46] to proline significantly increases sensitivity for GnRH-I, whereas mutations of a single amino acid residue at these positions does not affect ligand selectivity to GnRH-I, suggesting that the combination of each amino acid in TMH7 is critical for differential ligand selectivity.

On the basis of Millar's classification (Millar et al., 2004), we aligned the sequences of EL3 and proximal TMH7 of various GnRHR subtypes: human-1 and rat-1 for mammalian type I receptors, green monkey-2 and marmoset-2 for type II mammalian receptors, bullfrog-3 and *Xenopus*-2 for nonmammalian type II receptors, Japanese medaka-1 and bullfrog-2 for type III receptors, and finally Japanese medaka-2, bullfrog-2, and catfish-1 for nonmammalian type I receptors. Leu[7.40], Leu[7.44], and Asn[7.45] residues are con-

served between gmGnRHR-2 and the mammalian type I receptors, but these residues are also largely conserved in many other nonmammalian GnRHRs. Thus, these amino acid residues are not specific to mammalian GnRHR subtypes. After excluding the amino acids that are conserved throughout the GnRHR subtypes, we found that at least four residues, Iso$^{7.37}$, Leu$^{7.38}$, Gly$^{7.42}$, and Leu$^{7.43}$, in gmGnRHR-2 are different from those in mammalian type I receptors but are highly conserved in nonmammalian GnRHRs (Fig. 9). Among these, Iso$^{7.37}$ and Gly$^{7.42}$ are not likely to contribute to GnRH-I selectivity, because back-mutations of these residues in the rEL3L receptor did not significantly affect sensitivity to GnRH-I. Two amino acids, Ala$^{7.47}$ and Pro$^{7.48}$, in gmGnRHR-2 are different from either those in type I mammalian GnRHR or in nonmammalian GnRHRs. Thus, they are unique to the type II mammalian receptors.

Double or triple mutations of Leu$^{7.43}$, Ala$^{7.46}$, and Pro$^{7.47}$ in TMH7 significantly increase ligand sensitivity to GnRH-I. We cannot explain clearly how the combined mutation L$^{7.38}$F/A$^{7.46}$P/P$^{7.47}$C increases GnRH-I sensitivity. It is unlikely that these residues have direct interactions with GnRH-I because they are deeply buried in the three-dimensional structure. Rather, the mutation on these residues may play a role in modulating conformation of the binding pocket

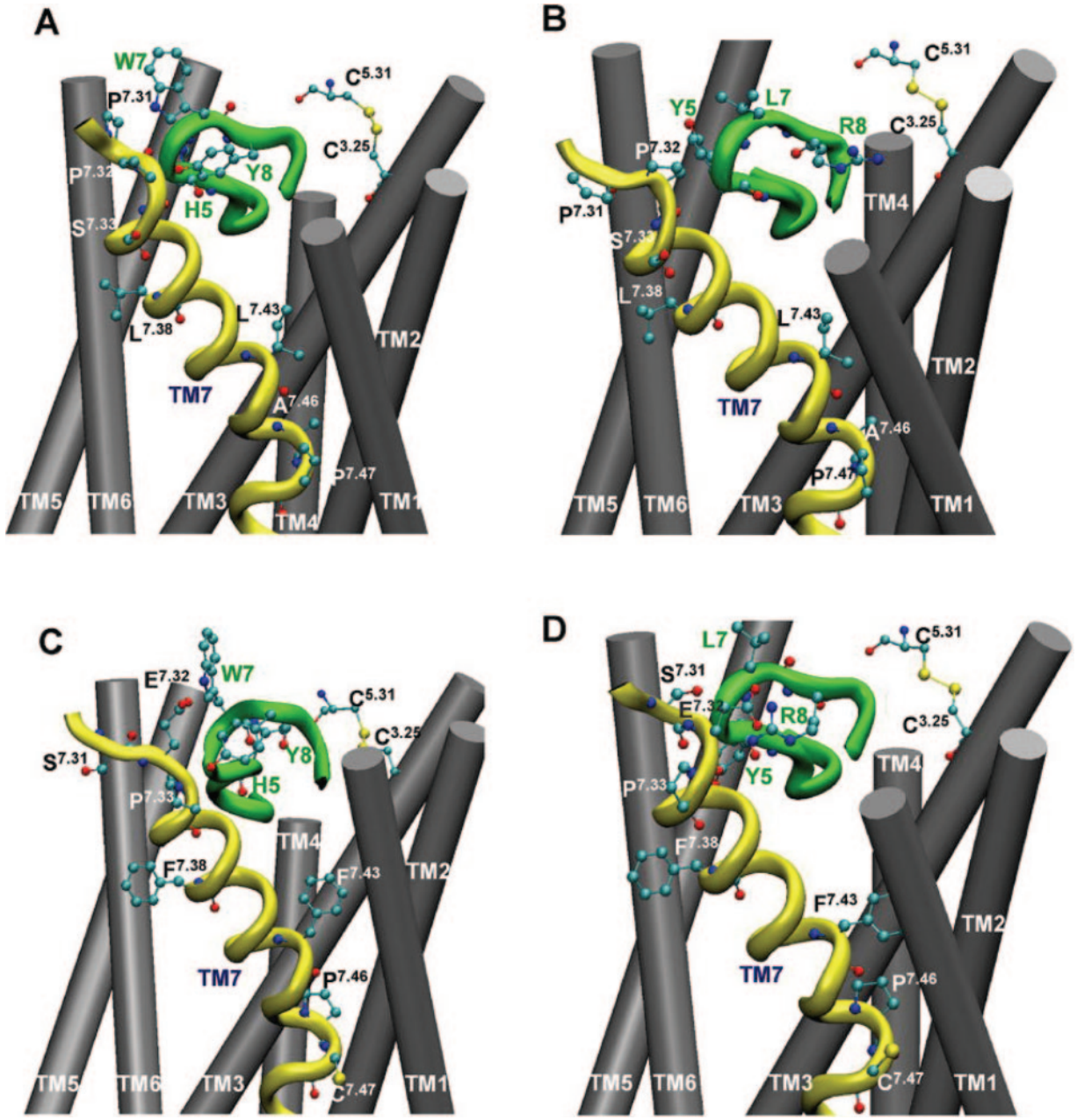


Fig. 8. Molecular models for the interaction of GnRH-II with wild-type gmGnRHR-2 (A), GnRH-I with wild-type gmGnRHR-2 (B), GnRH-II with the SEP/L$^{7.38}$F/L$^{7.43}$F/A$^{7.46}$P/P$^{7.47}$C mutant (C), and GnRH-I with SEP/L$^{7.38}$F/L$^{7.43}$F/A$^{7.46}$P/P$^{7.47}$C mutant (D). TMHs 1 to 6 of GnRHRs were drawn as cylinders in gray. TMH7 and ligands were drawn as tubes in yellow and green, respectively. The following residues were drawn as a ball-and-stick model with carbon atoms in cyan, oxygen atoms in red, nitrogen atoms in blue, and sulfur atoms in yellow. Pro/Ser$^{7.31}$, Pro/Glu$^{7.32}$, Ser/Pro$^{7.33}$, Leu/Phe$^{7.38}$, Ile/Phe$^{7.43}$, Ala/Pro$^{7.46}$, Pro/Cys$^{7.47}$, Cys$^{3.25}$, and Cys$^{4.78}$ of receptors, and His/Tyr^{5}, Trp/Leu^{7}, and Tyr/Arg^{8} of ligands are identified with numbers.

in EL3. Our molecular modeling data consistently show no direct interactions of these residues with the ligand. It is of interest to note that inter- and intramolecular interactions of the mutant receptor could be modified by the ligand type applied, indicating that conformational changes in these residues may be closely related to the alteration in the ligand binding pocket of EL3.

Amino acids at positions 7.46 and 7.47 in TMH7 seem critical for receptor conformation and stability. The mutation of $Ala^{7.46}$ to proline in wild-type gmGnRHR-2 and the mutation of $Pro^{7.46}$ to alanine or $Cys^{7.47}$ to proline in rEL3L impair receptor responsiveness. Extremely low binding of these mutant receptors to GnRH suggests that this impairment can be ascribed to the loss of binding activity or receptor stability. It is known that a proline residue leads to a local constraint on the polypeptide chain conformation because of its pyrrolidine ring structure. Thus, proline at a proper position in TMH7 seems to be important for receptor conformation/stability in wild-type and mutant gmGnRHR-2. The occurrence of two successive proline residues found in the rEL3L/$C^{7.47}P$ and $A^{7.46}P$ mutants might disrupt receptor conformation/stability because the loss of responsiveness of $A^{7.46}P$ mutant can be rescued by a double mutation ($A^{7.46}P/P^{7.47}C$). Furthermore, $Pro^{7.46}$ in the SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutant has an intramolecular contact with $Leu^{7.44}$, which is different from that of the wild-type receptor in which $Ala^{7.46}$ at the same position has a hydrophobic interaction with $Leu^{7.43}$. $Pro^{7.47}$ alone in gmGnRHR-2 could not critically alter receptor conformation/stability because the mutation of $Pro^{7.47}$ to cysteine did not affect ligand potency. The cysteine residue at position 7.47 is highly conserved in nonmammalian and mammalian type I GnRHRs, as well as in many other GPCRs, except for gmGnRHR-2 (Fig. 9). Thus, it may be possible that mutation of $Pro^{7.47}$ to cysteine helps to form a more stable conformation. Mutation of $Leu^{7.43}$ to phenylalanine in gmGnRHR-2 abolishes the receptor responsiveness to ligand, which can be rescued by a double mutation ($L^{7.43}F/P^{7.47}C$). In a three-dimensional structure, $Leu^{7.43}$ and $Pro^{7.47}$ are spatially very close. Therefore, it is postulated that a mutation of $Leu^{7.43}$ to phenylalanine might cause a steric hindrance, which can be reversed by a further replacement of $Pro^{7.47}$ to cysteine. Moreover, double mutations ($L^{7.43}F/P^{7.47}C$ or $A^{7.46}P/P^{7.47}C$) not only rescue the activity of the $L^{7.43}F$ or $A^{7.46}P$ mutant, but also significantly increase the ligand sensitivity to both GnRH-I and GnRH-II. Furthermore, an approximately 100-fold increase in sensitivity toward GnRH-I was observed in the triple mutant $L^{7.43}F/A^{7.46}P/P^{7.47}C$ compared with that of wild-type gmGnRHR-2. Such an increase in sensitivity to ligands suggests that this motif is crucially involved in receptor activation. Thus, it is likely that $Pro^{7.47}$ in the wild-type gmGnRHR-2 and $Pro^{7.46}$ in mutant receptors are involved in TMH movements, contributing to GPCR activation/inactivation by forming molecular hinges or swivels (Sansom and Weinstein, 2000; Stitham et al., 2002).

It is noteworthy that replacement of EL3 and EL3-proximal TMH7 or mutations of amino acids in these regions did not decrease sensitivity to GnRH-II; instead, there was a slightly increased sensitivity to GnRH-II. It is well known that an acidic amino acid at position 7.32 in EL3 is required for high-affinity binding with Arg^8 of GnRH-I. It seems that such an acidic residue also plays a certain role in interaction with Tyr^8 of GnRH-II. Using a molecular model, Blomenröhr et al. (2002) suggested that Tyr^8 of GnRH-II interacts with $Glu^{7.32}$ in EL3 of the catfish GnRHR. Our molecular model also consistently showed that Tyr^8 of GnRH-II has contact with $Glu^{7.32}$ of the SEP/$L^{7.38}F/L^{7.43}F/A^{7.46}P/P^{7.47}C$ mutant. It should be noted that Arg^8 of GnRH-I did not interact with the PPS motif of wild-type gmGnRHR-2, whereas Tyr^8 of GnRH-II had contact with $Pro^{7.32}$ of wild-type gmGnRHR-2.

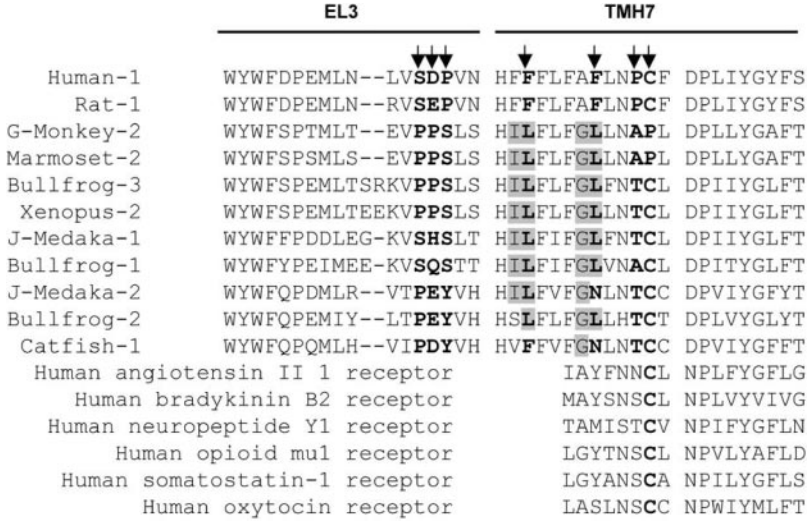


Fig. 9. Amino acid sequence alignment of EL3 and TMH7 in GnRHRs and other GPCRs. The amino acids in EL3 and TMH7 responsible for ligand selectivity are shown in boldface type and are indicated by arrows. The amino acids that are highly conserved among mammalian type II and nonmammalian GnRHRs but differ from the mammalian type I receptor are highlighted. It is noteworthy that the cysteine residue at position 7.48 is highly conserved in other GPCRs. Sequences used are human-1 GnRHR (GenBank accession no. NM_000406), rat-1 GnRHR (GenBank accession no. NM_031038), green (G) monkey-2 GnRHR (GenBank accession no. AF353988), marmoset-1 GnRHR (GenBank accession no. AF368286), *Xenopus*-2 GnRHR (GenBank accession no. AF257320), bullfrog-1–3 GnRHR (GenBank accession nos. AF144063, AF153913, and AF144062), Japanese (J) medaka-1–2 GnRHR (GenBank accession nos. AB057677 and AB057676), catfish-1 GnRHR (GenBank accession no. X97497), human angiotensin II 1 receptor (GenBank accession no. AF245699), human bradykinin B2 receptor (GenBank accession no. NM_000623), human neuropeptide Y1 receptor (GenBank accession no. NM_000909), human opioid μ1 receptor (GenBank accession no. NM_001008505), human somatostatin-1 receptor (GenBank accession no. NM_001049), and human oxytocin receptor (GenBank accession no. AY389507).

Thus, at least in the gmGnRHR-2 structure, GnRH-II may not discriminate the receptor with the PPS motif from the receptor with the SEP motif.

Substitution of histidine for Tyr^5 of GnRH-I did not alter its potency to activate wild-type gmGnRHR-2, rEL3L, or SEP/$L^{7.38}$F/$L^{7.43}$F/$A^{7.46}$P/$P^{7.47}$C, suggesting that position 5 of GnRH does not largely contribute to receptor-ligand interaction. This result is consistent with previous reports (Blomenröhr et al., 2002; Wang et al., 2003). Substitution of tryptophan for Leu^7 or Arg^8 in GnRH-I significantly increased the ability to activate gmGnRHR-2, indicating the importance of positions 7 and 8 in recognition of mammalian type II GnRHR. The SEP/$L^{7.38}$F/$L^{7.43}$F/$A^{7.46}$P/$P^{7.47}$C mutant showed ligand sensitivity similar to that of rEL3L, implying that multiple residues are required for distinguishing GnRH-II from GnRH-I. It is noteworthy that gmGnRHR-2 shows a high sequence identity in EL3-proximal TMH7 with nonmammalian GnRHRs but a relatively low sequence identity with that of mammalian type I GnRHR (Fig. 9). The evolutionary divergence of EL3 and TMH7 between mammalian types I and II GnRHR, therefore, may confer the differential selectivity toward GnRH-I and GnRH-II.

In summary, our studies demonstrate that EL3 and EL3-proximal TMH7 are responsible for differential ligand selectivity between mammalian types I and II GnRHRs. The elucidation of specific domains responsible for ligand selectivity may facilitate the understanding of ligand and receptor molecular coevolution, the mechanism of ligand-mediated GnRHR activation, and the development of novel drugs.

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Address correspondence to: Dr. Jae Young Seong, Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Republic of Korea. E-mail: jyseong@jnu.ac.kr