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Receptor-selective determinants in catfish gonadotropin seat-belt loops

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Abstract

Mammalian gonadotropins are highly selective. Charge differences between the Cys^{10–11} sequence of FSH β and LH β /CG β seat-belt loops determine the ability of these hormones to interact with the LH-R. Selective FSH-R binding is mainly dependent on the presence of an FSH β -specific sequence between Cys^{11–12} of the seat-belt loop. Intriguingly, African catfish LH β (cfLH β) lacks a positively charged Cys^{10–11} region and stimulates both catfish LH-R and FSH-R with comparable potencies. Our studies on the promiscuous behaviour of cfLH using chimeric gonadotropins revealed that the Cys^{10–11} region of cfLH β contains cfLH-R-selective determinants, whereas the Cys^{11–12} region of cfLH β confers FSH-R-stimulating activity to cfLH. Hence, the location of receptor-selective determinants appeared to be fairly well conserved throughout evolution, despite the low sequence identity between mammalian and catfish seat-belt loops. Moreover, various structure–function differences between gonadotropins are discussed in the context of the different (female) reproductive strategies between mammalian and non-mammalian species that required the divergence to a more specific LH-R-stimulating activity of one of the gonadotropins in mammals. © 2004 Elsevier Ireland Ltd. All rights reserved.

Keywords: Luteinizing hormone; Follicle-stimulating hormone; Hormone selectivity

1. Introduction

In mammals, gonadal function is regulated by two distinct, but complementary acting, pituitary-derived gonadotropins (follicle-stimulating hormone, FSH, and luteinizing hormone, LH) via the specific activation of their respective receptors (FSH-R and LH-R). LH and FSH, together with thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG), form the family of glycoprotein hormones, which are glycosylated heterodimeric molecules, each composed of a non-covalent association of a common α -subunit with a hormone specific β -subunit. Apart from LH and CG that both are able to activate the LH-R in primates and equids, the normal interaction between the glycoprotein hormones and

their respective receptors are highly specific (<0.1% cross-reactivity; Campbell et al., 1997; Vischer et al., 2003a). In contrast to mammalian gonadotropins, their counterparts in fish display reduced receptor selectivity. For example, purified, pituitary-derived catfish LH (cfLH) as well as recombinant cfLH (rcfLH) can activate both the catfish LH receptor (cfLH-R; Vischer and Bogerd, 2003a) and catfish FSH receptor (cfFSH-R; Bogerd et al., 2001) with comparable potencies. However, cfLH is not able to activate the catfish TSH-R (Vischer and Bogerd, 2003b). Moreover, recombinant catfish FSH (rcfFSH) predominantly activates the cfFSH-R (Vischer et al., 2003b).

Crystallographic analyses of deglycosylated hCG and hFSH (Lapthorn et al., 1994; Fox et al., 2001) suggested a similar overall folding of the common α -subunit, but also of the two gonadotropin β -subunits, each consisting of a cystine knot architecture that divides each subunit into three elongated antiparallel loops (i.e. α 1, α 2, α 3; β 1, β 2, β 3). Although the primary β -subunit sequences have diverged suffi-

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ciently during evolution to confer specificity to each of these heterodimeric glycoprotein hormones (Li and Ford, 1998), their β -subunits are structurally very similar due to the positional conservation of 12 conserved cysteine residues. Six cysteines form the cystine knot motif, while the remaining six residues form three additional loop-stabilizing disulfide bridges.

Based on sequence differences between distinct β -subunits, chimeric analogs have been generated in order to identify β -subunit regions that confer receptor-specificity to these hormones. These studies revealed that the region between the 10th and 12th conserved cysteine residues (Cys^{10–12}; i.e. the seat-belt region) of each glycoprotein hormone β -subunit is critically involved in determining specificity for its respective receptor. In particular, the net charge differences in the determinant loop (i.e. the region between Cys¹⁰ and Cys¹¹) of the seat-belt region between mammalian LH/CG β -subunits on the one hand, and FSH/TSH β -subunits on the other hand are thought to have partially separated LH-R- from FSH-R/TSH-R-activating properties (Han et al., 1996; Campbell et al., 1997). It is thought that additional sequence divergence in the carboxy-terminal seat-belt segment (i.e. between Cys¹¹ and Cys¹²) as well as outside the seat-belt region has further separated specific lutropic, follitropic and thyrotropic activities of these hormones to their respective natural receptors (Grossmann et al., 1997).

In contrast to the situation in mammalian gonadotropins, inspection of the determinant loop of fish gonadotropins revealed the absence of striking net charge differences between their LH and FSH β -subunits. To elucidate if and how the seat-belt region of cflH β contributes to the observed promiscuous receptor activation (Bogerd et al., 2001; Vischer and Bogerd, 2003a), we studied the effects of several substitutions of the intercysteine segments of the cflH β seat-belt region in order to identify sequence information necessary to specifically stimulate the cfFSH-R and the cflH-R.

2. Material and methods

2.1. Construction and expression of cassette-substituted gonadotropin cDNAs

Chimeric seat-belt cflH β -subunit (cflH β) constructs were generated by cassette-substitution of individual intercysteine segments of the seat-belt region or the entire seat-belt region between the 10th and 12th conserved cysteine residues (i.e. Cys⁹⁰ and Cys¹⁰⁷, respectively) with the corresponding hCG-regions or cfFSH-regions, or with the corresponding number of Ala residues (Fig. 1). In addition, reciprocal chimeras were made by introducing the cflH seat-belt region sequences into the cfFSH β -subunit (cfFSH β) (Fig. 1). To this end, the cDNAs encoding the cfFSH β (GenBank accession no. AF324541) or the cflH β (GenBank accession no. X97761), in which the first 30 bases of their leader sequences were optimized for *Dictyostelium discoideum* codon

	C10		C11		C12
cflH β	C	TMDTSD	C	TIESLNPDF	C
cfFSH β	C	NTEITD	C	GAFSMQPSS	C
cflH/Ala ^{10–11}	C	AAAAAD	C	TIESLNPDF	C
cflH/Ala ^{11–12}	C	TMDTSD	C	AAAAAAAAA	C
cflH/Ala ^{10–12}	C	AAAAAD	C	AAAAAAAAA	C
cflH/FSH ^{10–11}	C	NTEITD	C	TIESLNPDF	C
cflH/FSH ^{11–12}	C	TMDTSD	C	GAFSMQPSS	C
cflH/FSH ^{10–12}	C	NTEITD	C	GAFSMQPSS	C
cflH/hCG ^{10–11}	C	RRSTTD	C	TIESLNPDF	C
cflH/hCG ^{11–12}	C	TMDTSD	C	GGPKDHPLT	C
cflH/hCG ^{10–12}	C	RRSTTD	C	GGPKDHPLT	C
cfFSH/LH ^{10–11}	C	TMDTSD	C	GAFSMQPSS	C
cfFSH/LH ^{11–12}	C	NTEITD	C	TIESLNPDF	C
cfFSH/LH ^{10–12}	C	TMDTSD	C	TIESLNPDF	C

Fig. 1. Wild type and mutant cflH and cfFSH β -subunit seat-belt amino acid sequences. The amino acid sequences of the seat-belt region between the conserved Cys¹⁰ and Cys¹² of cflH β and cfFSH β are shown. Substitutions of the determinant loop (between Cys¹⁰ and Cys¹¹) and/or C-terminal segment (between Cys¹¹ and Cys¹²) of the seat-belt region of cflH β with Ala-cassettes (cflH/Ala), corresponding cfFSH β (cflH/FSH) or hCG β (cflH/hCG) segments are shown, of which the mutated segments are underlined. In addition, cfFSH β /cflH β (cfFSH/LH) chimeras are shown.

usage, and preceded by a *Dictyostelium* consensus translation initiation sequence (AAAAA; Vervoort et al., 2000) and flanked by *Bgl*III and *Spe*I restriction endonuclease sites at their 5'- and 3'-ends, respectively (Vischer et al., 2003b), were used as templates for PCR-based mutagenesis (Ali and Steinkasserer, 1995). Briefly, two cDNA fragments were generated using sense and antisense mutation primers in combination with specific primers demarcating the 3'- and 5'-end of the open-reading frame, respectively, on the above mentioned templates. Next, these two PCR products were used in a self-primed fusion PCR reaction. The fusion products were subsequently PCR amplified using specific primers demarcating the open-reading frame. All PCR products generated were TOPO-cloned into pcDNA3.1/V5-His plasmid (Invitrogen) for sequence analysis. The mutant seat-belt β -subunit cDNA inserts were then transferred into the *Dictyostelium* extra-chromosomal expression vector MB12neo, using their *Bgl*III and *Spe*I endonuclease restriction sites (Linskens et al., 1999).

Mutant gonadotropins were recombinantly expressed in the soil amoeba *Dictyostelium discoideum* (strain AX3) and purified as described previously (Vischer et al., 2003b). Briefly, 1 μ g of MB12n vector, containing the catfish glycoprotein hormone α -subunit cDNA (GenBank accession no. X97760; cGPa/MB12n), in combination with 1 μ g of mutant β -subunit/MB12neo circular plasmids were cotransformed in 10^7 cells by electroporation. Next, the cells were seeded in 9 cm culture plates. Selection for the α -subunit-containing construct with Blastidicin S (10 μ g/ml; ICN, Zoetermeer, The Netherlands) was initiated 5 h after electroporation. After 24 h, the medium was replaced

with fresh medium containing both Blasticidin S (10 mg/ml) and neomycin antibiotic G418 (5 mg/ml; Invitrogen), selecting cells harboring both the cfGP α /MB12n and β -subunit/MB12neo plasmids. Medium was refreshed every 3–4 days, while maintaining selective conditions. Approximately 6 ml of medium was harvested every 3.5 days, in total 3–4 times, starting 10–14 days after electroporation. Harvested medium was stored at -80°C until used for experimentation.

The recombinant catfish mutant gonadotropins were ~ 20 -fold concentrated by ultrafiltration, while simultaneously replacing the axenic *Dictyostelium* medium by DMEM-Hepes (Sigma, St. Louis, MI), using Ultrafree-15 Biomax-30 centrifugal devices (Amicon, Millipore Corporation, Bedford, MA). Hormone concentrations were quantified by radioimmunoassays using antisera against intact cLH and the cfGP α -subunit as described previously (Schulz et al., 1995).

2.2. *In vitro* gonadotropin receptor activation

In vitro bioactivities of the chimeric catfish gonadotropins were determined on human embryonic kidney T 293 (HEK-T 293) cells expressing the cfFSH-R or the cLH-R, as described previously (Bogerd et al., 2001; Vischer and Bogerd, 2003a). Briefly, approximately 5×10^6 HEK-T 293 cells were transiently cotransfected with 10 μg of a pCRE/ β -gal reporter-gene construct containing a β -galactosidase gene driven by five cAMP responsive elements (Chen et al., 1995) and either 1 μg of the human LH-R (kindly provided by Dr. E. Milgrom, Institut National de la Sante et de la Recherche Medicale, La Kremlin-Bicetre, France), 1 μg of the cfFSH-R or 10 ng of the cLH-R expression vector constructs. One-hundred-fold less expression vector construct of the constitutively active cLH-R compared with the cfFSH-R was used for transfection. This reduces the cLH-R-mediated basal cAMP levels allowing the dose-response curves to fit within the measuring range of the reporter-gene assay (Vischer and Bogerd, 2003a). After 16–18 h, cells were transferred to 96-well plates ($\sim 2.5 \times 10^5$ cells/well). The next day, the transfected cells were stimulated for 6 h with various concentrations of each of the mutant catfish gonadotropins in 25 ml DMEM-Hepes containing 0.1% BSA (Sigma). The ligand-induced β -galactosidase activity was measured, and the hormone concentrations inducing half-maximal stimulation (EC_{50}) were calculated using the Graphpad PRISM3 software package (GraphPad Software Inc., San Diego, CA). All experiments were repeated at least 3 times using cells from independent transfections and recombinant hormones derived from different batches.

2.3. Statistics

All results are expressed as the mean $\pm$ S.E.M. To evaluate the effect of increasing hormone concentrations on cLH-R- or cfFSH-R-mediated signaling over basal, the β -galactosidase activity was subjected to analysis of variance

(ANOVA) using Statview 4.5 (Abacus Concepts, Berkeley, CA). The ANOVA was followed by a Fisher's probable least-squares difference test to identify significant differences ($P < 0.05$) between individual groups. The same analysis or the student *t*-test ($P < 0.05$) was used to compare the log-transformed half-maximal stimulation (EC_{50}) of the cfFSH-R- or cLH-R-mediated signal transduction, induced by the recombinant catfish gonadotropins.

3. Results

3.1. Catfish gonadotropin expression in *Dictyostelium*

Recombinant mutant gonadotropins, expressed in *Dictyostelium discoideum*, were first functionally analyzed (see below), and a posteriori quantified by radioimmunoassay in order to limit the storage time between the concentration procedure and the functional assays. Recombinant gonadotropin levels were measured by two different radioimmunoassays using either a polyclonal antibody against intact cLH, or a polyclonal antibody against the cfGP α -subunit, as described previously (Vischer et al., 2003b). Typically, the amount of wild type cLH was $\sim 2.2 \pm 0.11$ -fold lower in the cfGP α radioimmunoassay than in the intact cLH radioimmunoassay, as cfGP α contributes to approximately half of the total molecular weight of intact heterodimeric cLH (Koide et al., 1992; Rebers et al., 1997). However, all chimeric cLH analogs, in particular cLH/Ala^{11–12}, cLH/Ala^{10–12}, cLH/hCG^{11–12} and cLH/hCG^{10–12}, had intact hormone/cfGP α ratios lower than 2.2 (Table 1). Since less than 0.1% of free cfGP α subunit is recovered after ultrafiltration (data not shown), low intact hormone/cfGP α ratios are likely to reflect mutation-induced alterations in the overall heterodimeric analog conformation, rather than a surplus of free cfGP α subunits due to impaired subunit association. In the absence of a specific cfFSH radioimmunoassay, the recombinant cfFSH and cfFSH analog levels were calculated from the anti-cfGP α measurements as described previously (Vischer et al., 2003b), without taking into account possible mutation-induced conformational changes. For convenience, all recombinant wild type and chimeric catfish LH and FSH amounts were quantified using the cfGP α measurements and corrected for their heterodimeric stoichiometry.

3.2. Bioactivities of the chimeric recombinant catfish gonadotropins

The bioactivities of the chimeric hormones were evaluated by measuring their potency to induce specific receptor-mediated signaling in HEK-T 293 cells, transiently transfected with either the cLH-R or the cfFSH-R. To examine whether promiscuous receptor binding of cLH is confined to one or both of the two intercysteine modules of its β -subunit seat-belt loop, the individual β -subunit Cys^{10–11} and Cys^{11–12} regions as well as the entire β -subunit seat-belt re-

Table 1

Summary of the intact cflH/cfGP α ratios and bioactivities of wild type cflH and cfFSH and mutant β -subunit seat-belt cflH and cfFSH analogs on HEK-T 293 cells, transiently expressing the cflH-R or the cfFSH-R

Hormone analogs	Seat-belt sequence		RIA cflH/cfGP α	cflH-R cAMP EC ₅₀ (ng/ml)	cfFSH-R cAMP EC ₅₀ (ng/ml)
	C ¹⁰ –C ¹¹	C ¹¹ –C ¹²			
rcfLH	cflH	cflH	2.2	45.50 $\pm$ 14.46 ^a	7.61 $\pm$ 1.29 ^b
cflH/Ala ^{10–11}	Ala	cflH	1.13	>300*	3.03 $\pm$ 1.05 ^b
cflH/Ala ^{11–12}	cflH	Ala	0.36	NS	>100*
cflH/Ala ^{10–12}	Ala	Ala	0.46	NS	>100*
cflH/hCG ^{10–11}	hCG	cflH	1.72	19.55 $\pm$ 4.83 ^a	1.59 $\pm$ 0.30 ^c
cflH/hCG ^{11–12}	cflH	hCG	0.92	>100*	>300*
cflH/hCG ^{10–12}	hCG	hCG	0.05	>300*	NS
cflH/FSH ^{10–11}	cfFSH	cflH	1.95	>300*	0.41 $\pm$ 0.07 ^c
cflH/FSH ^{11–12}	cflH	cfFSH	1.54	>300*	0.70 $\pm$ 0.11 ^c
cflH/FSH ^{10–12}	cfFSH	cfFSH	1.75	>300*	0.13 $\pm$ 0.13 ^c
rcfFSH	cfFSH	cfFSH		>300*	0.29 $\pm$ 0.04 ^c
cfFSH/LH ^{10–11}	cflH	cfFSH		NS	NS
cfFSH/LH ^{11–12}	cfFSH	cflH		>1000*	1.70 $\pm$ 0.28 ^c
cfFSH/LH ^{10–12}	cflH	cflH		>1000*	NS

Recombinant gonadotropin levels were measured using two different RIAs as described under Section 2. The ratio between the amounts of intact cflH hormone and cfGP α was used as an index to assess hormone conformation, with ratios lower than 2.2 suggesting an mutation-induced impaired hormone conformation. The cAMP production upon stimulation with wild type or mutant cflH and cfFSH analogs was measured in HEK-T 293 cells transiently cotransfected with either the cflH-R or the cfFSH-R expression vector construct and a plasmid (pCRE/ β -gal) containing a β -galactosidase gene under control of a promoter containing five cAMP-response elements. Due to the limited amounts of analogs produced by *Dictyostelium discoideum* and due to reduced activities of certain analogs, it was not always possible to calculate their half-maximal stimulation (EC₅₀). Hence, analog concentrations that were at least required to induce half-maximal stimulation, were estimated for these cases and are indicated by an asterisk. All data represent an average of at least three independent receptor-stimulation assays with triplicate observations each, and EC₅₀ values are given as mean $\pm$ S.E.M. ^{a,b,c}Values sharing the same letter do not differ significantly (see Section 2). NS: no significant stimulation of receptor-mediated cAMP production.

gion (Cys^{10–12}) were replaced with Ala-residues excluding the cysteine residues Cys¹⁰, Cys¹¹ and Cys¹². Since the Asp-residue preceding the 11th Cys residue is highly conserved in all glycoprotein hormones and has been shown to be crucial for their activity (Chen et al., 1991; Grossmann et al., 1997), this residue (i.e. Asp⁹⁶ in cflH β) was also excluded in the mutagenesis procedure to create the chimeric gonadotropins (Fig. 1). Replacing Cys^{10–11} of the cflH β seat-belt with Ala-residues (cflH/Ala^{10–11}) significantly reduced its cflH-R-stimulating activity, but did not affect its cfFSH-R-stimulating activity (Fig. 2A and Table 1). However, Ala-cassettes substitutions between Cys^{11–12} (cflH/Ala^{11–12}) and the entire seat-belt (cflH/Ala^{10–12}), substantially reduced the cflH-R- and cfFSH-R-stimulating activities for both types of chimeric hormones.

To determine more conclusively whether the native seat-belt sequence of cflH β is important for its cfFSH-R-stimulating activity, cflH analogs were generated that harbor seat-belt sequences of hCG. Since hCG had been shown to activate the cflH-R (Vischer and Bogerd, 2003a), but not the cfFSH-R (Bogerd et al., 2001), the resulting cflH/hCG chimeras were expected to maintain their cflH-R-stimulating activity, while being devoid of any cfFSH-R-stimulating activity. Surprisingly, however, substitution of the Cys^{10–11} sequence of cflH β with the corresponding sequence of hCG β (cflH/hCG^{10–11}) increased the cfFSH-R-stimulating activity of the cflH chimera to a potency that is similar to rcfFSH,

whereas its cflH-R-stimulating activity was not affected (Fig. 2B and Table 1). Introduction of the hCG β Cys^{11–12} region into cflH β (cflH/hCG^{11–12}) resulted in at least 40- and 2-fold reduced cfFSH-R- and cflH-R-stimulating activities, respectively (Fig. 2B and Table 1). Substitution of the entire seat-belt with hCG β sequences (cflH/hCG^{10–12}) abolished all measurable cfFSH-R-stimulating activity, while its cflH-R-stimulating activity was significantly attenuated (Fig. 2B and Table 1). Neither rcfLH nor the cflH/hCG chimeras were able to induce hLHR-mediated cAMP production over basal (data not shown).

In addition, the seat-belt segments of cflH β were substituted with the corresponding sequences of cfFSH β . Recombinant cfFSH had been shown to have a significantly lower potency for the cflH-R compared with cflH (Vischer et al., 2003b). Substitution of the cflH β seat belt with the entire cfFSH β seat-belt sequences (cflH/FSH^{10–12}), or the Cys^{10–11} and Cys^{11–12} intercysteine segments of cfFSH β (cflH/FSH^{10–11} and cflH/FSH^{11–12}, respectively) resulted in a significant reduction of their cflH-R-stimulating activities comparable to the intrinsic activity of rcfFSH to stimulate the cflH-R (Fig. 2C and Table 1). Their abilities to activate the cfFSH-R, on the other hand, were increased to potencies similar to wild type rcfFSH (Fig. 2C and Table 1). In contrast, all reciprocal chimeras, in which cfFSH β seat-belt segments were replaced by their corresponding cflH β sequences (i.e. cfFSH/LH^{10–11}, cfFSH/LH^{11–12} and cfFSH/LH^{10–12}), ac-

Fig. 2. Bioactivity of mutant cfLH and cfFSH β -subunit seat-belt analogs to induce cAMP production of cfLH-R and cfFSH-R, transiently expressed in HEK-T 293 cells. Effects of wild type recombinant cfFSH and cfLH, cfLH/Ala mutants (A), cfLH/hCG chimeras (B), cfLH/FSH chimeras (C) and cfFSH/LH chimeras (D) on the cAMP-mediated reporter gene activity in HEK-T 293 cells, transiently transfected with cfLH-R (left panel) or cfFSH-R (right panel). Results are shown as the mean $\pm$ S.E.M. of triplicate observations from a single representative experiment. Mean EC_{50} values are presented in Table 1.

tivated the cfLH-R in a similar way as wild type rcfFSH (Fig. 2D and Table 1). Only cfFSH/LH^{11–12} was able to significantly induce a receptor-mediated cAMP response in cfFSH-R expressing cells (Fig. 2D and Table 1), and had a similar potency to stimulate the cfFSH-R as rcfFSH.

4. Discussion

Glycoprotein hormones have coevolved together with their cognate receptors through gene duplications of ancestral β -subunit and receptor genes, respectively, followed by se-

quence divergence to define hormone-receptor pair selectivity (Moyle et al., 1994; Li and Ford, 1998; Moyle et al., 1998). However, functional duality of gonadotropins has not always evolved simultaneously with their structural divergence. To investigate if and how the seat-belt loop of cflH β allows the interaction of cflH with both the cflH-R and cfFSH-R, we systematically substituted the two successive cflH β intercysteine sequences in the seat-belt loop (i.e. sequences between the conserved Cys residues 10–11 and 11–12) with Alacassettes, or the corresponding cfFSH β or hCG β sequences. Replacing amino acid side chains with an Ala methyl-moiety would reveal if the seat-belt loop of cflH β is involved in directing cfFSH-R and/or cflH-R recognition, whereas substitutions with corresponding cfFSH β and hCG β regions would be informative on how the β -subunit seat belt confers receptor specificity to cflH. It should be noted that cfFSH as well as hCG are very specific in stimulating the cfFSH-R or cflH-R, respectively (Vischer et al., 2003b; Vischer and Bogerd, 2003a). In addition, we introduced cflH β seat-belt loops into the cfFSH β to investigate if they can confer cflH-R and/or maintain cfFSH-R stimulating-activity to chimeric cfFSH analogs.

Concentrations of recombinant catfish gonadotropin analogs were determined by two different radioimmunoassays using polyclonal antisera against either the common cfGP α or intact cflH. Typically, substitution of the Cys^{11–12} region of cflH β with Ala and hCG β residues resulted in cflH analogs with lower intact hormone/cfGP α radioimmunoassay ratios as compared to wild type cflH, suggesting that their overall conformation may have changed as a result of the mutations. This notion was supported by the fact that substitution of the cflH β Cys^{11–12} region with corresponding FSH β sequences did not severely alter the intact hormone/cfGP α ratio, indicating that the cflH seat belt itself does not form a major epitope for anti-intact cflH antisera binding. Typically, the overall conformation of human TSH/FSH/CG and equid CG analogs appeared not to be affected by interchange and/or mutagenesis of their respective seat-belt loops (e.g. Moyle et al., 1994; Dias et al., 1994; Lindau-Shepard et al., 1994; Grossmann et al., 1997; Chopineau et al., 2001). Crystal structures of hCG and hFSH revealed that the Cys^{11–12} region of their β -subunit seat-belt loops are tightly wrapped around the third loop of the α -subunit (i.e. α L3; Laphorn et al., 1994; Fox et al., 2001). Since the overall cflH conformation appeared to be rather sensitive to substitutions of the Cys^{11–12} region with Ala cassettes and corresponding hCG sequences, one may speculate that only the Cys^{11–12} region of cflH β and cfFSH β are tolerated in the vicinity of the α L3 of the cflH analogs.

Ala-cassette substitution of the determinant loop of cflH β revealed that the determinant loop residues of cflH β (i.e. amino acids TMDTS, excluding D⁹⁶; see Fig. 1) is indispensable for cflH-R-stimulating activity, but seems not to be involved in conferring cfFSH-R-stimulating activity to cflH. The importance of the Cys^{10–11} region of cflH β for its cflH-R-stimulating activity was further supported by the

substitution with its cfFSH β counterpart. In addition, the introduction of the cfFSH β determinant loop into cflH resulted in an $\sim$ 18.5-fold increase in its cfFSH-R-stimulating activity compared to wild type cflH, revealing the presence of important cfFSH-R-selective determinants in this region of cfFSH β . In contrast, except for the conserved Asp residue (i.e. amino acid residue 93 of human FSH β ; see Fig. 3), the determinant loop of human FSH β is not essential for FSH binding to the hFSH-R, but restrains the interaction with the hLH-R (Lindau-Shepard et al., 1994; Campbell et al., 1991; Moyle et al., 1994). The determinant loop of hCG β , on the other hand, and in particular its net positive charge, was found to be crucial for LH-R-stimulating activity, without influencing FSH-R-recognition (Dias et al., 1994; Han et al., 1996). Surprisingly, the introduction of the cflH β determinant loop in cfFSH β did not lead to an increased potency to stimulate the cflH-R. Moreover the cfFSH/LH^{10–11} analog was found to be devoid of both cfFSH-R- and cflH-R-stimulating activity. In view of the fact that wild type cflH displays intrinsic cfFSH-R-stimulating activity, it is unlikely that the cflH β determinant loop residues would be able to fully inhibit the potency to stimulate the cfFSH-R when introduced into cfFSH. Hence, we believe that the apparent absence of any bioactivity may be related to changes in overall conformation of cfFSH.

Replacing the cflH β determinant loop with the one of hCG β did not change the cflH-R-stimulating potency compared with wild type cflH. In contrast to the situation in hCG (Han et al., 1996), this result suggests that the net charge of the determinant loop is of minor importance in directing cflH-R-stimulating activity to cflH. Since the determinant loops of cflH β and hCG β share only a Thr residue, which corresponds to an Ile residue in cfFSH β (Fig. 1), our observations predict a role for this hydrophylic side chain in mediating specific cflH-R-stimulating activity. However, site-directed mutagenesis revealed that the corresponding Thr residue in hCG (i.e. Thr⁹⁷) is not involved in LH-R binding (Huang et al., 1993). Surprisingly, the hCG β determinant loop also led to an increased cfFSH-R-stimulating potency of this cflH analog. This contrasts earlier observations, in which the presence of the hCG β determinant loop in the context of human FSH β or TSH β did not enhance their interaction with the FSH-R or TSH-R, respectively, but rather conferred bifunctionality to these chimeric analogs (Dias et al., 1994; Grossmann et al., 1997). Taking into account the receptor-binding profiles of these chimeric human glycoprotein hormone analogs, the large heterogeneity in physicochemical properties between the determinant loop side chains of hCG β and cfFSH β (see Fig. 1), and in particular the fact that hCG is devoid of cfFSH-R-stimulating activity (Bogerd et al., 2001), it is difficult to explain how the determinant loops of hCG β and cfFSH β exert a similar effect on cfFSH-R-stimulating activity when placed in the context of cflH.

Thus, our findings suggest that the determinant loop of cflH β is essential for LH activity but seems to be dispensable for FSH activity. In this respect, the function of the de-

	C10		C11		C12															
shark LHβ	C	R	M	D	Y	T	D	C	T	V	Q	S	I	K	P	D	F	-	-	C
sturgeon LHβ	C	R	M	E	S	S	D	C	T	I	Q	G	V	G	P	S	D	-	-	C
goldfish LHβ	C	T	M	D	T	S	D	C	T	I	E	S	L	Q	P	D	F	-	-	C
Atlantic salmon LHβ	C	N	M	D	T	S	D	C	T	I	E	S	L	Q	P	E	F	-	-	C
African catfish LHβ	C	T	M	D	T	S	D	C	T	I	E	S	L	N	P	D	F	-	-	C
striped bass LHβ	C	A	M	D	T	S	D	C	T	F	E	S	L	Q	P	N	F	-	-	C
Australian lungfish LHβ	C	K	L	D	Y	T	D	C	T	V	Q	S	I	G	A	D	F	-	-	C
newt LHβ	C	K	M	D	Y	S	D	C	T	V	Q	S	I	G	P	E	F	-	-	C
bullfrog LHβ	C	K	M	D	Y	S	D	C	T	V	E	S	S	E	P	D	V	-	-	C
chicken LHβ	C	P	M	A	T	S	D	C	T	V	Q	G	L	G	P	A	F	-	-	C
ostrich LHβ	C	P	M	A	T	A	D	C	T	V	A	G	L	G	P	A	F	-	-	C
rat LHβ	C	R	L	S	S	S	D	C	G	G	P	R	T	Q	P	M	T	-	-	C
human LHβ	C	R	R	S	T	S	D	C	G	G	P	K	D	H	P	L	T	-	-	C
human CGβ	C	R	R	S	T	T	D	C	G	G	P	K	D	H	P	L	T	-	-	C
donkey LH/CGβ	C	R	L	K	T	T	D	C	G	G	P	R	D	H	P	L	A	-	-	C
horse LH/CGβ	C	Q	I	K	T	T	D	C	G	V	F	R	D	Q	P	L	A	-	-	C
shark FSHβ	C	N	T	E	T	T	D	C	T	V	S	A	M	E	P	T	H	-	-	C
sturgeon FSHβ	C	A	T	D	Y	T	D	C	G	T	L	S	L	G	P	S	D	-	-	C
goldfish FSHβ	C	N	S	D	I	T	D	C	G	V	L	S	Q	Q	T	L	G	-	-	C
Atlantic salmon FSHβ	C	E	T	D	N	T	D	C	D	R	I	S	M	A	T	P	S	-	-	C
African catfish FSHβ	C	N	T	E	I	T	D	C	G	A	F	S	M	Q	P	S	S	-	-	C
striped bass FSHβ	C	N	T	E	N	T	D	C	G	R	F	P	E	D	I	P	S	-	-	C
Australian lungfish FSHβ	C	H	T	E	T	T	D	C	T	V	G	G	L	G	P	S	Y	-	-	C
newt FSH β	C	D	T	D	H	T	D	C	T	V	R	G	L	G	P	N	Y	-	-	C
bullfrog FSHβ	C	D	S	E	T	T	D	C	T	V	R	A	L	G	P	T	Y	-	-	C
chicken FSHβ	C	D	T	D	S	T	D	C	T	V	R	G	L	G	P	S	Y	-	-	C
ostrich FSHβ	C	D	T	D	S	T	D	C	T	V	R	G	L	G	P	S	Y	-	-	C
rat FSHβ	C	D	S	D	S	T	D	C	T	V	R	G	L	G	P	S	Y	-	-	C
human FSHβ	C	D	S	D	S	T	D	C	T	V	R	G	L	G	P	S	Y	-	-	C
horse FSHβ	C	N	S	D	S	T	D	C	T	V	R	G	L	G	P	S	Y	-	-	C
sturgeon TSHβ	C	N	T	D	Y	S	E	C	T	M	E	P	L	R	P	S	P	-	-	C
goldfish TSHβ	C	N	T	N	S	D	E	C	A	H	K	T	N	N	A	G	M	-	K	C
Atlantic salmon TSHβ	C	N	T	D	S	D	E	C	A	H	K	A	S	S	G	D	G	A	R	C
Australian lungfish TSHβ	C	N	T	D	T	T	D	C	I	N	G	A	E	A	T	I	Q	-	-	C
chicken TSHβ	C	N	T	D	Y	S	D	C	V	H	E	K	V	R	T	N	Y	-	-	C
rat TSHβ	C	N	T	D	Y	S	D	C	T	H	E	A	V	K	T	N	Y	-	-	C
human TSHβ	C	N	T	D	Y	S	D	C	I	H	E	A	I	K	T	N	Y	-	-	C

Fig. 3. Alignment of amino acid sequences of the seat-belt region of LH/CG, FSH and TSH β-subunits of species from various classes. Conserved cysteine residues are indicated by black boxes, whereas the conserved aspartic acid in the determinant loop is depicted in bold and italics. In addition, positively charged residues are indicated by grey boxes, while negatively charged residues are underlined.

terminant loop of fish LHβ appeared to be similar as that of mammalian LHβ/CGβ, directing the hormone's activity towards the LH-R. In human gonadotropins, the net charge difference between their determinant loops determines the ability of hCG and hFSH to interact with the LH-R (Campbell et al., 1991; Moyle et al., 1994; Han et al., 1996). Accurate comparison of superimposed crystal structures of hCG and hFSH revealed significant differences in surface charge characteristics between the hFSHβ and hCGβ determinant loops (Fox et al., 2001): three Asp residues in FSH form a negatively charged patch on one side of the determinant loop. On the other hand, the positively charged Arg residues in the determinant loop of hCGβ are not arranged as a charged patch, as their side chains are directed to opposite sides of the loop. The negatively charged surface of the determinant loop of hFSHβ is not essential for binding to the hFSH-R, but acts

as an inhibitory determinant by blocking unintended interactions with hLH-R (Lindau-Shepard et al., 1994; Dias et al., 1994). Typically, the determinant loop of cfFSHβ appeared to be essential for cfFSH-R-stimulating activity, but seemed not to be actively involved in inhibiting the interaction with cfLH-R. Since determinant loops of fish LH and FSH share a similar negative net charge and positional conservation of acidic residues, of which the side chains are predicted to have a similar spatial orientation (cf. crystal structures of hCG and hFSH; Laphorn et al., 1994; Fox et al., 2001), the mechanism by which key determinants in determinant loops of fish gonadotropins confer receptor-stimulating activity to these hormones remains puzzling.

The primary sequence of the Cys^{11–12} loop of cfLHβ seems to be important for both cfFSH-R- and cfLH-R-stimulating activity as indicated by Ala-cassette substitu-

tion. However, Ala-cassette mutation affected the potency to stimulate the cLH-R more than its cFSH-R stimulating activity. In contrast, replacing the Cys^{11–12} residues of cLH β with the corresponding hCG β sequences affected the cFSH-R-stimulating activity more than its potency to stimulate the cLH-R. Similarly, introducing the Cys^{11–12} residues of hCG β into human FSH β significantly attenuated FSH-R-binding activity, however, without adding any LH-R-stimulating activity to this FSH analog (Dias et al., 1994). Nonetheless, it should be taken into account that substitution of the Cys^{11–12} residues of cLH β with Ala residues or the corresponding hCG β residues may have changed the overall cLH analog conformation, so that a direct effect of these mutations on receptor interaction can not be undoubtedly distinguished from an indirect effect resulting from altered hormone conformation. However, the fact that cLH/Ala^{11–12} and cLH/hCG^{11–12} had comparable cFSH-R-stimulating activities, supports the importance of the primary sequence of the Cys^{11–12} region of cLH β in conferring cFSH-R selectivity. The potency of cLH to stimulate the cFSH-R was significantly increased by replacing the Cys^{11–12} residues of cLH β with the corresponding cFSH β residues. Remarkably, however, the Cys^{11–12} region of cLH β was sufficient to confer full cFSH-R-stimulating activity when placed in the context of cFSH.

Although the Cys^{11–12} loop sequence has diverged considerably during the course of evolution between most LH/CG and FSH β -subunit paralogs, and in particular between orthologous β -subunits of fish and tetrapods (see Fig. 3), this region appeared to be of utmost importance for FSH activity in all species tested. Placing the human FSH β Cys^{11–12} loop in the context of hCG, allowed this hCG analog to interact with the FSH-R (Campbell et al., 1991; Moyle et al., 1994), whereas substituting the FSH β Cys^{11–12} loop with the corresponding hCG β loop significantly impaired the binding to the FSH-R (Dias et al., 1994). Subsequent Ala-scanning analysis delineated the amino acid triplet Arg-Gly-Leu as being crucial for FSH-R binding (Lindau-Shepard et al., 1994). Likewise, the potency of horse LH/CG (eLH/CG; also known as pregnant mare serum gonadotropin) to promiscuously activate nonequine FSH-R, but not the horse FSH-R, relies exclusively upon the sequence of eCG/LH β Cys^{11–12} loop (in particular Val-Phe-Arg; Chopineau et al., 2001). Typically, this equine-specific triplet can transfer additional FSH activity to donkey LH/CG, but was not sufficient enough to confer FSH-R-stimulating activity to hCG.

Based on the property to bind both FSH-R and LH-R, Moyle and co-workers (Campbell et al., 1991; Moyle et al., 1994; Han et al., 1996; Moyle et al., 1998) proposed that an hCG β chimera that harbors its native, positively charged determinant loop in conjunction with the hFSH β Cys^{11–12} region represents an omnipotent ancestral β -subunit. According to this model, LH β /CG β -like hormones and FSH β /TSH β -like hormones have evolved from this common ancestor, predominantly through sequence divergence of

their seat-belt loops. In particular the introduction of negative charges into the determinant loop of FSH β /TSH β -like hormones would have attenuated their LH-R binding affinities. In contrast to this model, non-mammalian gonadotropins (i.e. LH β and FSH β) have net negatively charged determinant loops (Fig. 3). Together with the observed receptor promiscuity of chicken LH (Wakabayashi et al., 1997), bullfrog LH (Takada et al., 1986), and cLH, phylogenetic data suggest that a presumed ancestral β -subunit had a negative charge and that neutrally or positively charged determinant loops were acquired during mammalian LH β /CG β evolution. Moreover, in contrast to the situation in mammals, our results indicate that charge differences in the determinant loop do not form the basis of the observed receptor selectivity in the catfish, which may rather reside in differences in hydrophobicity of the determinant loop.

Duality in gonadotropins is already established in elasmobranch fishes (Quérat et al., 2001), which have emerged before the divergence between actinopterygians (i.e. chondrosteans and bony fish) and sarcopterygians (i.e. lungfishes and tetrapods) approximately 400 million years ago (Hedges and Kumar, 2003). Although phylogenetic analysis clusters all FSH, LH, and TSH β -subunit orthologs in separate branches (Vischer et al., 2003c; Quérat et al., 2004), striking differences are present between orthologous β -subunits when looking at their seat-belt loops (Fig. 3). Characteristic differences are the number of acidic residues in the determinant loop and the divergence in Cys^{11–12} loop sequence between actinopterygian and sarcopterygian FSH β -subunits. However, more significant are the net charge differences in the determinant loop and the differences in Cys^{11–12} loop sequence between non-mammalian and mammalian LH/(CG) β -subunits. Of particular interest is the high sequence identity in the Cys^{11–12} region between LH β and FSH β of amphibian and avian species, which may be associated with the observed FSH-R binding activity of chicken and bullfrog LH (Takada et al., 1986; Wakabayashi et al., 1997). Interestingly, the divergence between non-mammalian and mammalian LH β /(CG β) seat-belt loops seems to coincide with the necessity of a highly specific LH/CG, which came forth from a drastic change in female reproduction strategy. Whereas non-mammalian vertebrates are predominantly oviparous (i.e. females deposit unfertilized or developing eggs that complete their development and hatch in the external environment), mammals are generally viviparous characterized by an extended intrauterine pregnancy. The maintenance of an intrauterine pregnancy depends on a prolonged production of progesterone by the corpus luteum in response to LH/CG. In the ovary, primordial follicles grow spontaneously and randomly to become primary follicles that express FSH-R on their granulosa cells (Fauser and Van Heusden, 1997). In order to avoid undesired folliculogenesis during pregnancy as a result of the promiscuous activation of the FSH-R by elevated LH/CG levels, high receptor selectivity of mammalian LH/CG is an absolute requirement.

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