

Evidence that the α -subunit influences the specificity of receptor binding of the equine gonadotrophins

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Abstract

Horse LH/chorionic gonadotrophin (eLH/CG) exhibits, in addition to its normal LH activity, a high FSH activity in all other species tested. Donkey LH/CG (dkLH/CG) also exhibits FSH activity in other species, but about ten times less than the horse hormone. In order to understand the molecular basis of these dual gonadotrophic activities of eLH/CG and dkLH/CG better, we expressed, in COS-7 cells, hybrids between horse and donkey subunits, between horse or donkey α -subunit and human CG β (hCG β), and also between the porcine α -subunit and horse or donkey LH/CG β . The resultant recombinant hybrid hormones were measured using specific FSH and LH *in vitro* bioassays which give an accurate measure of receptor binding specificity and activation. Results

showed that it is the β -subunit that determines the level of FSH activity, in agreement with the belief that it is the β -subunit which determines the specificity of action of the gonadotrophins. However, donkey LH/CG β combined with a porcine α -subunit exhibited no FSH activity although it showed full LH activity. Moreover, the hybrid between horse or donkey α -subunit and hCG β also exhibited only LH activity. Thus, the low FSH activity of dkLH/CG requires an equine (donkey or horse) α -subunit combined with dkLH/CG β . These results provide the first evidence that an α -subunit can influence the specificity of action of a gonadotrophic hormone.

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Introduction

The glycoprotein hormone family consists of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone, which are secreted by the pituitary gland in all mammalian species, and chorionic gonadotrophin (CG), which is secreted by the placenta in primates and equids. These hormones are composed of a common α -subunit and a hormone-specific β -subunit that is commonly thought to confer hormonal specificity (Pierce & Parsons 1981). Unlike primates which have a family of CG β genes derived from an ancestral LH β gene (Talmadge *et al.* 1984), horses and donkeys have only a single gene encoding their LH and CG β -subunits (Stewart & Maher 1991, Sherman *et al.* 1992, Chopineau *et al.* 1995), which is therefore referred to as the LH/CG β -subunit gene.

Equine gonadotrophins have unique biological properties: horse LH (eLH) and horse CG (eCG) are basically LH molecules but express very high LH and FSH activities in non-equids; donkey LH (dkLH) and donkey CG (dkCG) behave similarly but have much less FSH activity than eLH and eCG (Aggarwal *et al.* 1980, Roser *et al.* 1984). Recombinant eLH and dkLH (r.eLH/CG and r.dkLH/

CG) have recently been expressed in COS-7 cells and their activities assessed in two *in vitro* bioassays (Chopineau *et al.* 1997). These recombinant hormones exhibit the same characteristics as the natural hormones: if the FSH/LH ratio is set at 1 for r.eLH/CG it is 0.1 for r.dkLH/CG (Chopineau *et al.* 1997), showing that the low FSH activity of dkLH/CG is intrinsic to the molecule and is not due to FSH contamination as some authors had previously suggested (Roser *et al.* 1984, Matteri *et al.* 1987). It is important to note, however, that these recombinant hormones are neither LH nor CG as the pattern of glycosylation in COS-7 cells is probably different from those in pituitary or placental cells. However, this does not appear to alter the FSH/LH ratios of the hormones.

The dual gonadotrophic activities of equine LH/CG in other species constitute a very good model to study the structure–function relationships of gonadotrophins. Hence it is possible to modify these hormones and follow the effects of the changes on one activity (FSH activity in this study), using the other activity (LH activity) as a control of the structural integrity of the hormone. In this study, we expressed recombinant hybrids between the horse and donkey subunits, between the porcine α -subunit and the

horse or donkey LH/CG β -subunit, and between the horse or donkey α -subunit and the human CG β -subunit, in order to delineate more precisely the structural origin of the dual LH/FSH activities of horse and donkey LH/CG.

Materials and Methods

Plasmid constructs

The cDNAs encoding the horse α -subunit, the donkey α -subunit, the horse LH/CG β -subunit and the donkey LH/CG β -subunit were inserted into the mammalian expression vector pCDM8 (InVitrogen, Leeks, The Netherlands) as already described (Chopineau *et al.* 1997).

The cDNA encoding the porcine α -subunit was cloned by RT-PCR from pituitary RNA using two specific primers based on the known cDNA sequence of this subunit (Kato *et al.* 1991), each including an XbaI restriction site for cloning (5'-TCTAGACTGCAA AAAATCCAGAGGAAGAAGA-3' as forward primer and 5'-TCTAGAAGTCATCAACAAGGCCCTCTGC AAA-3' as reverse primer). Sequencing in both directions by the dideoxy chain termination method using double-stranded DNA showed that the sequence of the cDNA was identical to that published previously (Kato *et al.* 1991). The cDNA was then inserted into pCDM8 by its XbaI site and the correct orientation detected by restriction mapping.

The cDNA encoding the human CG β -subunit was a kind gift from Dr J J Remy (INRA, Jouy en Josas, France).

Expression vector plasmids were amplified in MC1061/P3 bacteria (InVitrogen) and then purified using the Qiagen maxiprep kit (Coger, Paris, France).

Transient transfections of COS-7 cells

COS-7 cells were co-transfected at 65% of confluency in 6 cm diameter Petri dishes with 2 μ g of each vector, one containing an α -subunit (e α , dk α or porcine α (p α) and the other a β -subunit (eLH/CG β , dkLH/CG β or human CG β (hCG β)) using a calcium phosphate precipitation procedure as previously described (Chopineau *et al.* 1997). Control cells were transfected with the expression vector containing no insert (CDM8). After 48-h incubation in serum-free Dulbecco's modified Eagle's medium, media corresponding to each transfection (control CDM8, r.eLH/CG, r.dkLH/CG, r.e α /dkLH/CG β , r.dk α /eLH/CG β , r.p α /eLH/CG β , r.p α /dkLH/CG β , r.e α /hCG β and r.dk α /hCG β) were harvested, centrifuged and supernatants were collected and stored at -20 °C until assayed.

Quantification of recombinant dimers

The quantities of r.eLH/CG, r.dkLH/CG, r.e α /dkLH/CG β and r.dk α /eLH/CG β in the harvested media were estimated using a dimer-specific sandwich ELISA that

utilizes two monoclonal antibodies directed against eCG (Chopineau *et al.* 1993, 1997). This assay is very specific for the equine dimeric hormones and, as expected, it did not detect the hybrids between the porcine α -subunit and equine LH/CG β -subunits or the equine α -subunits and hCG β . However, as this study was primarily concerned with the FSH activity of these hybrids, their LH activities were used as a reference, so that the levels of FSH activity of the recombinant hybrids were related to their LH activities.

Estimation of LH and FSH bioactivities

The LH bioactivity of the recombinant hormones was estimated by measuring their ability to stimulate testosterone production in a rat Leydig cell bioassay (Guillou *et al.* 1985). The FSH bioactivity was assessed in an *in vitro* progesterone stimulation assay using Y1 cell line from a mouse adrenal cortex tumour stably expressing the human FSH receptor (gift from Ares Sero, Geneve) as already described (Chopineau *et al.* 1997).

The equine recombinant dimers in the harvested expression media were measured in the two assays directly (Table 1) and also after quantification by ELISA (Table 2). Dimers containing one non-equine subunit only were measured directly (Table 1).

Results and Discussion

The horse and donkey LH/CG molecules constitute an excellent model for the study of gonadotrophin structure-function relationships, as they are highly homologous (α -subunit=92% identity (Chopineau & Stewart 1996), β -subunit=93% identity (Sherman *et al.* 1992, Chopineau *et al.* 1995)) but differ in their relative FSH and LH bioactivities, dkLH/CG having an FSH/LH ratio ten times lower than eLH/CG (Chopineau *et al.* 1997). Furthermore, our recent experiments showing that it is possible to express the cDNAs to obtain biologically active gonadotrophins with the same FSH/LH ratios as the native hormones (Chopineau *et al.* 1997) has now progressed to the successful production of recombinant hybrids between various subunits, to provide a particularly powerful method for these investigations.

The β -subunit determines the level of FSH activity

The LH and FSH activities and FSH/LH ratios of the horse/donkey hybrid gonadotrophins are shown in Table 1 according to dilutions of the harvested expression media, and in Table 2 according to the quantification in the sandwich ELISA. First, the medium from cells transfected with the expression vector containing no insert (CDM8) did not stimulate either testosterone production in rat Leydig cells, or progesterone production in Y1 cells expressing human FSH receptors. Second, whereas the

Table 1 The FSH and LH activities (and calculated FSH/LH ratios) of recombinant-derived hybrid gonadotrophins resulting from the co-transfection (in COS-7 cells) of various combinations of *α*- and *β*-subunits: horse and donkey *α*- and *β*-LH/CG, horse or donkey *α*- and hCG-*β*, and porcine *α*- and horse or donkey LH/CG-*β*

Subunit		FSH activity		LH activity		FSH/LH	
		(a)	(b)	(a)	(b)	(a)	(b)
<i>α</i>	<i>β</i>						
—	—	0	0	0	0		
e	e	100	100	100	100	1	1
dk	e	40	17	33	14.5	1.2	1.17
e	dk	10	9	75	54	0.13	0.17
dk	dk	10	7	140	64	0.07	0.11
e	h	0	0	100	52	0	0
dk	h	0	0	51	70	0	0
p	dk	0	0	100	89	0	0

Each hybrid was measured according to dilutions of the harvested medium and *α*e/*β*eLH/CG was given a reference activity of 100%. The activities of the other recombinant hormones were calculated according to their ED₅₀ compared with *α*e/*β*eLH/CG. The results of two different expression experiments are given ((a) and (b)). An activity of 0 means that it was undetectable in the assay conditions (the limit of detection being 5 ng/ml *α*e/*β*eLH/CG in each assay). —, expression vector CDM8 alone without insert. Note that the porcine *α*/donkey LH/CG-*β* hybrid had no detectable FSH activity but full LH activity, resulting in a ratio of 0.

Table 2 The FSH and LH activities (and FSH/LH ratios) of the horse and donkey *α*- and *β*-LH/CG subunit hybrids, based on initial quantification by immunoassay

Subunit		FSH activity		LH activity		FSH/LH	
		(a)	(b)	(a)	(b)	(a)	(b)
<i>α</i>	<i>β</i>						
e	e	100	100	100	100	1	1
dk	e	70	48	58	41	1.2	1.17
e	dk	21.5	19	161	108	0.13	0.17
dk	dk	9	12	130	102	0.07	0.11

Each hybrid was tested according to an initial quantification using the equine-specific sandwich ELISA described in the text. The *α*e/*β*eLH/CG hybrid was given a reference activity of 100% in both assays. The activities of the other recombinant hormones were calculated according to their ED₅₀ compared with *α*e/*β*eLH/CG. The results of two different expression experiments are given ((a) and (b)).

relative activities were different in Tables 1 and 2, which reflects differences in the expressed quantities of the various hybrids, it is important to note that the calculated FSH/LH ratios were identical. This clearly illustrates that initial quantification of the hybrid hormones is not a critical point, and that the LH activity can be taken as a measure of the quantity of biologically active hormone present.

Our finding that the r.*α*/dkLH/CG-*β* hybrid exhibited the same FSH/LH ratio as r.dkLH/CG and that the r.dk*α*/eLH/CG-*β* hybrid had the same characteristics as the r.eLH/CG gave a very clear illustration that it is the *β*-subunit which determines the level of FSH activity, regardless of the *α*-subunit (donkey or horse). In other words, those molecules containing the dkLH/CG *β*-subunit exhibited a low FSH/LH ratio whereas those

containing the eLH/CG *β*-subunit gave a high FSH/LH ratio (Tables 1 and 2).

The bioactivities of the hetero-hybrids containing the hCG *β*-subunit are shown in Table 1. Both the horse and the donkey *α*-subunits successfully combined with the human *β*-subunit to give biologically active molecules with considerable LH activity but neither showed any detectable FSH activity.

In summary, whereas the LH activities were comparable for all the hybrids, the FSH activity was undetectable when the hybrid contained hCG-*β*, low when the hybrid contained the donkey LH/CG *β*-subunit and high when the hybrid contained the horse LH/CG *β*-subunit. These results show that it is the *β*-subunit which not only determines whether both LH and FSH activities are expressed, but also determines the level of FSH activity.

These results confirm many earlier studies, including the site-directed mutagenesis experiments with human gonadotrophins showing that substitution of residues 101–109 in hCG β by the corresponding amino acids of hFSH β results in a hormone with unchanged LH activity but with additional FSH activity (Campbell *et al.* 1991, Moyle *et al.* 1994). Furthermore, amino acid sequence analyses indicate that residues β 102 and 103 are probably the most important with regard to the high FSH activity of eLH/CG (Combarous 1992). These residues are Val-Phe in eLH/CG β , which is very similar to the Val-Arg doublet in all known FSHs and very different from the Gly-Pro doublet seen in all known LHs. However, dkLH/CG β also has Gly-Pro at these positions (and is not different from the other LHs at positions 101 and 104–109; Chopineau *et al.* 1995), therefore indicating that the structural origin of the low FSH activity of dkLH/CG resides in another part of the molecule.

Requirement of horse or donkey α -subunit to obtain FSH activity

To investigate if the low FSH activity of dkLH/CG was borne by its β -subunit, we co-expressed the donkey LH/CG β -subunit and the porcine α -subunit, a species whose LH is known to have no FSH activity. We also attempted to express a heterodimer between horse LH/CG β -subunit and porcine α -subunit.

While the r.p α /dkLH/CG β hybrid exhibited an LH activity similar to the LH activity of r.dkLH/CG, it exhibited no detectable FSH activity which is in marked contrast to r.dkLH/CG (Table 1). This result shows that replacing the donkey or horse α -subunit with the porcine α -subunit abolishes the FSH activity of dkLH/CG, at least on human FSH receptors. However, there is no evidence that the human FSH receptor differs from any of the other non-equine species with regard to specificity of recognition, and we can reasonably think that this hybrid exhibits no FSH activity in all non-equine species.

To our surprise, all attempts to obtain an active hybrid between the porcine α -subunit and eLH/CG β -subunit failed and we only ever obtained a small amount of LH activity (<10% of the other hybrids) and no detectable FSH activity (results not shown). This suggests that either an equine (horse or donkey) α -subunit is required for the expression of both the LH and FSH activities of eLH/CG or that the different species subunits did not combine very well. This result is in agreement with previous attempts to reassociate the equine subunits obtained from chemical dissociation of natural hormones. For example, attempts to recombine purified eCG β -subunit with the ovine α -subunit (Papkoff 1974) or with the human α -subunit (Christakos & Bahl 1979) did not result in any LH or FSH activities. Furthermore, hybrids between eLH β and ovine, porcine or human α -subunits were shown to have no detectable activity, although they did readily recombine as

assessed by SDS electrophoresis under non-dissociating conditions (Bousfield *et al.* 1985). It is clear from the results presented here that the low FSH activity of donkey LH/CG is attributable to a unique feature of this dimeric hormone and needs the association of its α -subunit and its β -subunit. The special characteristics of its α -subunit are shared with the horse α -subunit as the recombinant hybrid between horse α -subunit and donkey LH/CG β -subunit also exhibited low FSH activity. These two α -subunits have the same unusual C-terminus part when compared with all other α -subunits known, particularly an inversion between Tyr and His at positions 89 and 93, and Ile instead of Ser at position 96. Furthermore, deletion and mutagenesis studies have shown that this C-terminal part in the human α -subunit is important for bioactivity (Yoo *et al.* 1991, 1993, Chen *et al.* 1992). More interestingly, it has been shown that His 94 and Lys 95 of the α -subunit are important for high-affinity receptor binding and cyclic AMP (cAMP) production on hFSH stimulation whereas they are important for cAMP induction but significantly less important for high-affinity receptor binding of hCG (Zeng *et al.* 1995). Therefore, these two amino acids (94 and 95) play common and hormone-specific roles in receptor binding and receptor activation by hFSH and hCG. The fact that these two amino acids are flanked by unique amino acids in horse and donkey α -subunits compared with all other α -subunits known (i.e. positions 93 and 96) suggests that this region may favour an interaction with FSH receptors, provided the α -subunit has combined with horse or donkey LH/CG β -subunit.

With regard to horse LH/CG, its high FSH activity is most probably due to amino acids 102 and 103 within its β -subunit. In addition, the horse LH/CG β -subunit appears to differ from all other species tested in that it associates very poorly with α -subunits from other species. These results further demonstrate that the unusual properties of the equine gonadotrophins can be effectively exploited for the study of gonadotrophin structure and function.

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