Role of the Invariant Aspartic Acid 99 of Human Choriogonadotropin β in Receptor Binding and Biological Activity*

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The four human glycoprotein hormones are heterodimers that contain a common α subunit and a hormone-specific β subunit. Within this hormone family, 23 amino acid sequences from 11 mammalian species are available. There are 19 invariant amino acid residues in the β subunits, 12 of which are Cys that form six disulfide bonds. Of the remaining seven conserved amino acid residues, we have investigated the role of an Asp which occurs at position 99 in human choriogonadotropin β (hCGB). Site-directed mutagenesis was used to replace hCGB Asp99 with three residues, Glu, Asn, and Arg, and to prepare an inversion double mutant protein, Arg94→Asp and Asp99→Arg. The cDNAs were placed in a eukaryotic expression vector, and the plasmids were transiently transfected into Chinese hamster ovary cells containing a stably integrated gene for bovine α. Radioimmunoassays demonstrated that the mutant forms of hCGB were capable of subunit assembly to the same extent as hCGB wild type. The heterologous heterodimers were assayed in vitro using transformed mouse Leydig cells (MA-10) by competitive inhibition of 125I-hCG binding and stimulation of progesterone production. The gonadotropins containing Glu and Asn were active, although the potency was less than that associated with the hCGB wild type. The glycosylation, and the hormones, CG and LH, act through the same receptor, which has recently been cloned (McFarland et al., 1989; Loosfelt et al., 1989), while FSH and TSH each have their own receptors, which have also been cloned (Nagayama et al., 1989; Libert et al., 1989; Sprengel et al., 1990; Frazier et al., 1990; Misrahi et al., 1990). The β subunits of human LH, FSH, and TSH contain 111-114 amino acid residues, while that of CG is 145 amino acid residues in length, the additional size arising from a C-terminal extension not present in the pituitary hormones. Considerable amino acid sequence homology exists in the four β subunits and is greatest for CGβ and LHβ; these also share an N-terminal extension not found in FSHβ and TSHβ (Pierce and Parsons, 1981).

Alignment of the amino acid sequences of the β subunits shows that all 12 cysteines, which form six disulfide bonds, are conserved. In addition, seven other amino acid residues are conserved in the known mammalian amino acid sequences, or those deduced from cDNAs, of CGβ and LHβ (12 sequences from 10 species), FSHβ (six sequences), and TSHβ (five sequences) (Ward et al., 1990). The invariant residues in these 23 sequences, aside from the 12 cysteines, are Gly96, Tyr37, Pro78, Gly71, Val84, Ala86, and Asp99, using the numbering system for hCGB. The total conservation of these residues in four distinct gene products from 11 mammalian species argues for an important structural or functional role in chain folding, subunit assembly, receptor recognition (but not specificity), or receptor activation to produce a cellular response. It is noteworthy that the known forms of salmon gonadotropin β also contain Asp in the position equivalent to hCGB position 99 (cf. Ward et al., 1990).

The invariant Asp99 is located within the determinant loop, i.e. residues 93–100, proposed by Ward and co-workers (Moore et al., 1989) to be important in determining receptor specificity. Recently, Campbell et al. (1991) showed that a chimeric β subunit with amino acid residues 88–108 of FSHβ, corresponding to residues 94–114 of hCGβ, replacing residues 94–145 of hCGβ exhibited full FSH activity in vitro. Also, using site-directed mutagenesis to introduce premature termination codons in the DNA of hCGβ, we have shown that the 1–100 fragment associates with α to produce an active gonadotropin, albeit with reduced potency relative to the hormone containing a full-length β subunit (Chen and Puett, 1991a). In contrast, a 1–92 fragment failed to associate with α and was, of course, inactive. Thus, the region 93–100 of hCGβ appears to be critical for subunit assembly, perhaps as an important conformational determinant (Chen and Puett, 1991a), as well as for receptor binding.

In addition, we found that the mutant hCGB chain with an Arg94→Asp replacement, i.e. the corresponding residue in

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1 The abbreviations used are: CG, choriogonadotropin; CHOn, Chinese hamster ovary cells containing a stably integrated gene for bovine α; FSH, follitropin; h, human; LH, lutropin; TSH, thyrotropin.
hFSHβ, forms a heterodimer and is active, but with a reduced potency relative to the expressed hCGβ wild type-containing gonadotropin (Chen and Puett, 1991b). Earlier, Keutmann et al. (1989) prepared synthetic peptides of the 93–100 region and found that peptides based on the native sequence could block hCG binding to its receptor with an apparent ED50 of about 0.2 mM, but the peptide failed to produce a biological response. Replacement of certain residues with Asp greatly reduced the inhibitory effect on hCG binding to receptor. A similar peptide based on the hFSHβ amino acid sequence not only inhibited FSH binding to its receptor, but also stimulated steroidogenesis in granulosa cells (Santa Coloma and Reichert, 1990). Thus, the sequence encompassing residues 93–100 appears important in both subunit assembly and receptor recognition.

In this study we have used site-directed mutagenesis to replace Asp99 in hCGβ with three types of residues, a positively charged group, Arg, another negatively charged group, Glu, and a polar, sterically similar but non-ionizable group, Asn. In addition, an “inversion” mutant was prepared in which Arg99 and Asp99 were switched with each other. These mutant hCG β subunits were capable of forming inhibited heterodimer formation, but only the Glu and Asn replacements yielded active heterodimer in vitro, and these were of lower potency than that containing hCGβ wild type.

**EXPERIMENTAL PROCEDURES**

**Materials—**α-S-[32S]-dATP (500 Ci/mmol) and [1,2,6,7-3H]progesterone (94 Ci/mmol) were obtained from Du Pont-New England Nuclear; the progesterone antisera was purchased from Radioassay Systems Laboratories (Carson, CA). 125I-hCG (100–150 μCi/μg) and a polyclonal antisera against free and heterodimeric hCGβ were from ICN (Hormsha, PA), and the TANDEM RHCG kit, specific for hCG, was from Hybridtech (San Diego, CA). Mutagenic M13 (site-directed mutagenesis kit) was obtained from Bio-Rad, the Sequenase version 2 kit was from U. S. Biochemical Corp., and DH5α competent cells were from Bethesda Research Laboratories. Centricon 10 columns were purchased from Amicon (Danvers, MA) and P20 columns from 5-Prime-3-Prime, Inc. (Paoli, PA). Methotrexate was from Bristol Laboratories (Syracuse, NY), and ATP, lysozyme, and bovine serum albumin were obtained from Sigma.

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potent than Asn\textsuperscript{99}. Interestingly, these two mutant gonadotropins did not yield the same maximal stimulation as the \( \beta \) wild type-containing heterologous gonadotropin. Whether the apparent difference between the maximal progesterone stimulation elicited by the \( \beta \) replacements Glu\textsuperscript{99} and Asn\textsuperscript{99} is significant awaits further confirmation. Slight differences in maximal progesterone production were also observed in other steroidogenic assays, but significance was not reached. The heterologous gonadotropins containing Arg\textsuperscript{99} and Asp\textsuperscript{99}/Arg\textsuperscript{99}, i.e. the inversion mutant, were totally devoid of activity as assayed by progesterone production in MA-10 cells (Fig. 3B).

Preliminary data (not shown) on cAMP production in MA-10 cells (cf. Chen and Puett (1991a) were consistent with the competitive binding and progesterone results. Namely, the \( \beta \) Glu\textsuperscript{99} and Asn\textsuperscript{99} replacements yielded gonadotropins with reduced potency relative to that with hCG\( \beta \) wild type, while the \( \beta \) Arg\textsuperscript{99} replacement and the \( \beta \) inversion mutant, i.e. Arg\textsuperscript{99} \( \rightarrow \) Asp/Asp\textsuperscript{99} \( \rightarrow \) Arg, were inactive even at hormone concentrations of 10 ng/ml.

**DISCUSSION**

The extraordinary conservation of Asp\textsuperscript{99} in all known glycoprotein hormone \( \beta \) subunits suggests that this residue is of fundamental importance as a structural or functional unit in these heterodimeric hormones which exhibit varied biological activities. In addition to a potential critical role in determining protein conformation, such an invariant residue may also be important as a subunit or receptor contact site, or it may have an important role in signal transduction. Since there are three known receptors for this family of hormones (McFarland et al., 1989; Loosfelt et al., 1989; Sprengel et al., 1990; Frazier et al., 1990) yielding CG/LH, FSH, and TSH activity, Asp\textsuperscript{99} cannot be considered important in determining receptor specificity.

Our results using in vitro assays demonstrate that Asp\textsuperscript{99} can be substituted with other residues if negative charge is maintained, e.g. with Glu, or even comparable stereochemistry in a polar, but uncharged side chain, e.g. Asn, with the maintenance of some degree of bioactivity including receptor binding and signal transduction. If, however, a positive charge is placed at position 99, there is a loss of receptor binding; this also occurs in the inversion mutant, Arg\textsuperscript{99} \( \rightarrow \) Asp/Asp\textsuperscript{99} \( \rightarrow \) Arg. Hence, the requirement for Asp at position 99 is not absolute in hCG\( \beta \), and by inference not in the \( \beta \) subunits of LH/FSH/TSH as well, but of the three general classes of side-chain functional groups we surveyed, only the negatively charged and polar but uncharged groups yielded functional gonadotropins, albeit of reduced potency relative to the heterologous gonadotropin containing hCG\( \beta \) wild type. One can infer from these data that Asp\textsuperscript{99} may be involved in a charge-charge interaction with a positively charged residue on the receptor.

The approach of site-directed mutagenesis provides absolute specificity, but at this level it is hampered to the extent that one cannot readily distinguish direct effects mediated by side chain replacement, involving for example subunit or receptor contact from localized or even global conformational changes. In the case of the glycoprotein hormones, it can be assumed, with reasonable justification, that subunit assembly cannot proceed if global conformational changes occur. The validity of this statement can be documented by the various studies indicating that several noncontiguous regions compose the \( \alpha \beta \) contacts (Ward, 1978; Ji and Ji, 1981; Hwang and Menon, 1984). Moreover, disulfide formation within the sub-
units would probably not occur if significant conformational differences existed in the mutant proteins. From these arguments we conclude that the replacements we have made at Asp\(^9\) in hCG\(^\beta\) do not lead to large conformational changes; rather, the effects we find are attributed to direct receptor contact, or perhaps to rather localized conformational alterations.

There are, including Asp\(^9\), a total of 19 invariant amino acid residues in the 23 known mammalian amino acid sequences of CG/LH/FSH/TSH\(^\beta\). All 12 cysteines are invariant, and Asp\(^9\) is one of seven additional conserved non-Cys residues, the others being Gly\(^38\), Tyr\(^{37}\), Pro\(^{39}\), Gly\(^{71}\), Val\(^{44}\), and Ala\(^{48}\) (cf. Ward et al., 1990). Of these, Azuma et al. (1990) have recently studied the role of Gly\(^{36}\), part of the highly conserved Cys-Ala-Gly-Tyr sequence in \(\beta\) subunits, using site-directed mutagenesis. They found that replacement of Gly\(^{36}\) with either Asp or Arg led to negligible amounts of heterodimer formation. Thus, Gly\(^{36}\) appears to be an important determinant for subunit assembly. It is noteworthy that Ala\(^{58}\) is conserved in 22 of the 23 \(\beta\) sequences; in rat FSH\(\beta\) the corresponding residue is Glu (cf. Ward et al., 1990). Ala\(^{58}\) in hCG\(\beta\) was replaced with Asp, with no effect on the degree of subunit assembly, but no information was provided on bioactivity (Azuma et al., 1990). Thus, of the seven non-Cys invariant residues in the known mammalian CG/LH/FSH/TSH \(\beta\) amino acid sequences, we have shown that Asp\(^9\) is involved in receptor recognition, and Azuma et al. (1990) found that Gly\(^{38}\) is involved in recognition of the complementary subunit, assuming that their replacements did not result in \(\beta\) unfolding. The roles of the other invariant residues remain to be determined, although it has been suggested that Tyr\(^{37}\) is also involved in subunit assembly (Gordon and Ward, 1985).

Interestingly, a region contiguous to the Cys-Ala-Gly-Tyr sequence, residues 38–53 in hCG\(\beta\), appears to be an important determinant for receptor binding and activation. Keutmann et al. (1987, 1988) reported that a synthetic peptide corresponding to the amino acid sequence of hCG\(\beta\) residues 38–57 inhibits hCG binding to rat ovarian membranes with an ED\(_{50}\) of about 0.15 mM; in addition, the peptide stimulates testosterone production in rat Leydig cells (Keutmann et al., 1989). Receptor recognition by the synthetic peptide could be nullified by replacement of certain residues with Asp. Using site-directed mutagenesis we have shown that replacement of Arg\(^{43}\) with Asp, the corresponding residue in hFSH\(\beta\), did not alter subunit assembly, but biological activity was diminished, albeit not eliminated (Chen and Puett, 1991b). Replacement of Arg\(^{43}\) with Lys had no effect on subunit assembly or activity. We have also characterized an hCG\(\alpha\) mutant in which Arg\(^{43}\) was replaced with the corresponding residue in hFSH\(\alpha\), Ser; the mutant associated with \(\alpha\) and the resulting heterodimer was active, but with reduced potency relative to hCG\(\alpha\) (Xia et al., 1991). These findings strongly argue that several residues within the hCG\(\alpha\) 93–100 sequence are important in CG/LH receptor recognition, consistent with the proposal by Moore et al. (1980) that this sequence in the glycoprotein hormone \(\beta\) subunit is an important determinant for receptor specificity.

However, Campbell et al. (1991) found that substitution of hCG\(\alpha\) amino acid residues 94–114 with the corresponding sequence of hFSH\(\alpha\), with deletion of hCG\(\alpha\) residues 115–145, yielded a chimeric \(\beta\) subunit that associated with \(\alpha\) to yield a heterodimer with full FSH activity and no significant CG/LH activity in vitro. Moreover, they noted that two mutant hCG\(\beta\)s, with hFSH\(\beta\) sequences replacing those for hCG\(\beta\) at amino acid residues 94–97 and at 108–114, bound to \(\alpha\) to give CG/LH activity. Since the amino acid residues 98–100 are identical in hCG\(\alpha\) and hFSH\(\beta\), e.g. Thr-Asp-Cys, these results suggest an important role for residues 101–107 in FSH receptor binding. Coupled with the observation that the bovine \(\alpha\)des(101–145)hCG\(\beta\) heterodimer bound to the CG/LH receptor with less affinity than \(\alpha\)-hCG\(\alpha\) wild type (Chen and Puett, 1991a), it appears that residues located C-terminal to Cys 100 also contribute to CG/LH receptor recognition.

Approaches of this type with CG/LH and subunits, coupled with related structure-function information from chemical modifications (Gordon and Ward, 1985) and limited enzymatic cleavages (Birken et al., 1987; Bousfield and Ward, 1988), are providing critical information on the contribution of specific residues and regions to subunit assembly and receptor recognition/activation.

### REFERENCES


Role of Asp9 in hCGβ


