

# Asn<sup>102</sup> of the Gonadotropin-releasing Hormone Receptor Is a Critical Determinant of Potency for Agonists Containing C-terminal Glycinamide\*

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**We demonstrate a critical role for Asn<sup>102</sup> of the human gonadotropin-releasing hormone (GnRH) receptor in the binding of GnRH. Mutation of Asn<sup>102</sup>, located at the top of the second transmembrane helix, to Ala resulted in a 225-fold loss of potency for GnRH. Eight GnRH analogs, all containing glycinamide C termini like GnRH, showed similar losses of potency between 95- and 750-fold for the [Ala<sup>102</sup>]GnRHR, compared with wild-type receptor. In contrast, four GnRH analogs that had ethylamide in place of the C-terminal glycinamide residue, showed much smaller decreases in potency between 2.4- and 11-fold. In comparisons of three agonist pairs, differing only at the C terminus, glycinamide derivatives showed an 11–20-fold greater loss of potency for the mutant receptor than their respective ethylamide derivatives. Thus Asn<sup>102</sup> is a critical determinant of potency specifically for ligands with C-terminal glycinamide, while ligands with C-terminal ethylamide are less dependent on Asn<sup>102</sup>. These findings indicate a role for Asn<sup>102</sup> in the docking of the glycinamide C terminus and are consistent with hydrogen bonding of the Asn<sup>102</sup> side chain with the C-terminal amide moiety. Taken with previous data, they suggest a region of the GnRH receptor formed by the top of helices 2 and 7 as a binding pocket for the C-terminal part of the ligand.**

The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH)<sup>1</sup> acts via receptors on gonadotrope cells of the pituitary to control the release of luteinizing hormone and follicle-stimulating hormone. GnRH receptors (GnRHRs) cloned from mouse, rat, human, sheep, and cow are members of the G-protein-coupled receptor superfamily (1–7). The GnRHR is coupled via the G<sub>q</sub>/G<sub>11</sub> group of G-proteins to phospholipase C (8). Agonist activation of the GnRHR results in an oscillatory Ca<sup>2+</sup> signaling process (9) and gonadotropin exocytosis (10). Because of the central role of GnRH in reproductive physiology, a large number of peptide GnRH analogs has been synthesized, and both agonist and antagonist analogs have found clinical

applications in a range of disorders of reproductive function (11, 12). While nonpeptide analogs have been discovered for several heptahelical peptide receptors including the opioid (13) tachykinin (14), and angiotensin (15) receptors, nonpeptide GnRH analogs remain to be described. Detailed knowledge of the ligand binding site on the GnRHR is likely to facilitate rational design of both novel peptide and nonpeptide GnRH analogs.

Early studies showed that removal of the C-terminal glycinamide residue of GnRH led to a marked decrease in potency of the peptide (11), suggesting an important interaction of the glycinamide with the receptor. A modification that was found to enhance the *in vivo* potency of GnRH was the replacement of the glycinamide C terminus of the peptide with ethylamide (16): GnRH, pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-CO-NH-CH<sub>2</sub>-CO-NH<sub>2</sub>; [Pro<sup>9</sup>-NEt]GnRH, pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-CO-NH-CH<sub>2</sub>-CH<sub>3</sub>. The ethylamide modification was suggested to confer greater resistance to enzymatic degradation (11) and has been incorporated in most clinically used GnRH agonist analogs.

Site-directed mutagenesis provides a means for identifying receptor residues involved in ligand binding. In a range of G-protein-coupled receptors for peptide ligands, both the transmembrane helices and the extracellular domains have been shown to contain residues that are important in binding (17). When combined with systematic structural variation of the ligand, receptor mutagenesis has the potential to elucidate specific interactions between ligand and receptor. In a previous study investigating the role of glycosylation in the GnRHR, we found that Asn<sup>102</sup>, although present in a consensus *N*-glycosylation sequence, is not in fact glycosylated (18). Surprisingly, mutation of Asn<sup>102</sup> to Gln resulted in an increased potency of GnRH for the mutant receptor (18), which was due to increased binding affinity (data not shown). These findings suggested the possibility that Asn<sup>102</sup>, located at the top of transmembrane helix 2, might be directly involved in the ligand binding site. In the present study we have identified the side chain of Asn<sup>102</sup> of the GnRHR as an important determinant of agonist potency. Furthermore, we show that the enhancement of potency conferred by Asn<sup>102</sup> is selective for peptides with a glycinamide moiety at the C terminus, while the corresponding ethylamide derivatives are less dependent on Asn<sup>102</sup>, indicating an important role for this residue in the docking of the C-terminal glycinamide of the ligand.

## EXPERIMENTAL PROCEDURES

**Reagents and Peptides**—GnRH (pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH<sub>2</sub>), [His<sup>2</sup>,Trp<sup>7</sup>,Tyr<sup>8</sup>]GnRH (chicken II-GnRH), [Gln<sup>8</sup>]GnRH, [D-Trp<sup>6</sup>]GnRH, [D-Trp<sup>6</sup>,Pro<sup>9</sup>-NEt]GnRH were synthesized by

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<sup>1</sup> The abbreviations used are: GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; GnRH-OH, GnRH free acid.

TABLE I

Potencies of GnRH analogs for stimulation of inositol phosphate production in wild-type human GnRHR and [Ala<sup>102</sup>]GnRHR

EC<sub>50</sub> values are the mean ± S.E. from *n* independent experiments. The potency loss values shown are the mean ± S.E. of the ratio EC<sub>50</sub>(mutant)/EC<sub>50</sub>(wild-type) from *n* independent experiments. The nature of the C terminus of the peptide is indicated by G (glycinamide) or E (ethylamide).

PEPTIDE	WT-GnRHR EC <sub>50</sub>	[Ala <sup>102</sup> ]GnRHR EC <sub>50</sub>	Potency Loss EC <sub>50</sub> mut/EC <sub>50</sub> wt	<i>n</i>	G/E
	<i>nm</i>	<i>nm</i>			
1. GnRH	0.114 ± 0.030	24.1 ± 7.03	225 ± 41	4	G
2. [Pro <sup>9</sup> -NET]GnRH	0.144 ± 0.063	1.45 ± 0.72	11.1 ± 3.4	5	E
3. GnRH-OH	37.1 ± 12.2	4610 ± 1226	157 ± 76	3	
4. [D-Ala <sup>6</sup> ]GnRH	0.087 ± 0.022	10.8 ± 5.25	110 ± 25	4	G
5. [D-Ala <sup>6</sup> ,Pro <sup>9</sup> -NET]GnRH	0.028 ± 0.014	0.220 ± 0.075	9.59 ± 2.21	3	E
6. [D-Trp <sup>6</sup> ]GnRH	0.026 ± 0.005	0.510 ± 0.090	27.1 ± 12.4	5	G
7. [D-Trp <sup>6</sup> ,Pro <sup>9</sup> -NET]GnRH	0.034 ± 0.006	0.078 ± 0.015	2.42 ± 0.36	7	E
8. [Trp <sup>2</sup> ]GnRH	0.036 ± 0.003	3.43 ± 0.99	94.4 ± 23.5	3	G
9. [His <sup>5</sup> ,D-Tyr <sup>6</sup> ]GnRH	0.127 ± 0.031	74.2 ± 13.3	649 ± 150	4	G
10. [His <sup>5</sup> ,Trp <sup>7</sup> ,Tyr <sup>8</sup> ]GnRH	1.31 ± 0.77	167 ± 8.7	232 ± 88	3	G
11. [D-lys <sup>6</sup> ]GnRH	0.124 ± 0.055	30.2 ± 9.65	260 ± 37	2	G
12. [γ-lactam <sup>6,7</sup> ]GnRH	0.040 ± 0.010	4.30 ± 0.06	116 ± 29	2	G
13. [Gln <sup>8</sup> ]GnRH	2.78 ± 1.84	847 ± 298	748 ± 381	3	G
14. [Ala <sup>9</sup> ]GnRH	4.60	453	98	1	G
15. [D-Ser(Bu <sup>6</sup> ),Pro <sup>9</sup> -NET]GnRH	0.052 ± 0.008	0.321 ± 0.193	5.74 ± 2.83	2	E

conventional solid-phase methodology and purified by preparative C-18 reverse-phase high-pressure liquid chromatography. [Trp<sup>2</sup>]GnRH, [D-Ser(Bu<sup>6</sup>),Pro<sup>9</sup>-NET]GnRH (buserelin) and [γ-lactam<sup>6,7</sup>]GnRH were gifts from J. Rivier, J. Sandow (Hoechst) and R. M. Freidinger (Merck), respectively. GnRH free acid (GnRH-OH), [Pro<sup>9</sup>-NET]GnRH, [D-Ala<sup>6</sup>]GnRH, and [D-Ala<sup>6</sup>,Pro<sup>9</sup>-NET]GnRH were from Sigma. Peptide nomenclature follows that of Karten and Rivier (11).

**Receptor Constructs and Site-directed Mutagenesis**—The human GnRHR cDNA (5) was shortened by removal of 1.3 kilobases of the 3'-untranslated region by digestion with *Ssp*I and cloned into the *Eco*RV site of the phagemid pcDNA1/Amp (Invitrogen, San Diego, CA). Oligonucleotide-directed mutagenesis was performed using the method of Kunkel *et al.* (19). After passage through dut<sup>-</sup>ung<sup>-</sup>,F' strain *Escherichia coli* CJ236, uridine-containing antisense single-stranded DNA template was prepared using VCS M13 helper phage (Stratagene). A mutant oligonucleotide TGGGATGTGGGCCATTACAGTC was designed to encode the Ala<sup>102</sup> amino acid substitution. The oligonucleotide was phosphorylated with T4 polynucleotide kinase (Promega Corp.) and annealed with the template, and second strand synthesis was completed using T7 DNA polymerase (Bio-Rad) and T4 ligase (Bio-Rad). Products of the reaction were used to transform competent *E. coli* strain MV1109. Plasmid DNA from ampicillin-resistant colonies was sequenced, confirming the mutation, at the core facility of the Department of Biochemistry, University of Bristol, U.K. Two independent [Ala<sup>102</sup>]GnRHR clones, obtained from separate mutagenesis reactions, were subsequently shown to exhibit indistinguishable phenotypes.

**Transient Expression of GnRHRs**—Plasmid DNA for transfection was prepared using Qiagen columns (Qiagen) according to the manufacturer's instructions. COS-1 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal calf serum in a 10% CO<sub>2</sub> atmosphere. For inositol phosphate production assays, cells were seeded in poly-D-lysine-coated 12-well plates 1 day before transfection, at 1.8 × 10<sup>5</sup> cells/well. For binding studies, 3 × 10<sup>6</sup> cells were seeded into 90-mm diameter dishes. Cells were transfected by a modification of the DEAE-dextran method (20). Cells were washed twice with phosphate-buffered saline, pH 7.4, and then incubated with 0.5 ml/well or 4 ml/dish of serum-free Dulbecco's modified Eagle's medium containing 3.5–5 μg/ml plasmid DNA and 0.3 mg/ml DEAE-dextran for 4 h at 37 °C. This was replaced with Dulbecco's modified Eagle's medium containing 2% fetal calf serum and 150 μM chloroquine, and incubation was continued for 1 h at 37 °C. The cells were washed with phosphate-buffered saline, and finally cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum.

**Inositol Phosphate Production**—24 h after transfection, cells were labeled overnight with 2 μCi/ml *myo*-[2-<sup>3</sup>H]inositol (Amersham Corp.) in 0.25 ml/well Medium 199 (Life Technologies, Inc.) containing 5% fetal calf serum, washed twice in HEPES-buffered saline (buffer A; Ref. 21), and then stimulated with GnRH as indicated. Incubations were stopped with perchloric acid/phytic acid solution, and following neutralization with KOH, total inositol phosphates were chromatographed on Dowex columns as described previously (21).

**Data Reduction**—Peptide concentrations required to stimulate half-

maximal IP production (EC<sub>50</sub>) were estimated by nonlinear regression to the equation  $IP = IP_{max}/(1 + EC_{50}/A)$ , where IP is the inositol phosphate response to concentration *A* of agonist, and IP<sub>max</sub> is the maximal response, using Prism (Graphpad) software. Figures show representative experiments in which data points are the mean ± range of duplicate determinations. Table I shows mean ± S.E. of the indicated number of independent experiments.

## RESULTS

In COS-1 cells transiently expressing the [Ala<sup>102</sup>]GnRHR, the EC<sub>50</sub> for GnRH-stimulated inositol phosphate production was increased by a mean of 225-fold, compared with the wild-type receptor (Table I). In 15 independent experiments using the 15 peptide agonists shown in Table I, the mean maximal inositol phosphate response of the mutant receptor was 122% ± 26% of the wild-type value (mean ± S.D.). These results indicated that the mutant receptor was expressed normally and that the loss of potency of GnRH for the [Ala<sup>102</sup>]GnRHR can be attributed predominantly or entirely to decreased binding affinity. Decreases in receptor number or coupling can cause a large increase in EC<sub>50</sub> without a decrease in maximal inositol phosphate response only where there is a high degree of receptor reserve (22). This was not the case in our experimental system, since (a) GnRHR mutants, which have normal binding affinity but are poorly expressed, *e.g.*, glycosylation-defective mutants, showed large decreases in maximal inositol phosphate responses, with only small increases in EC<sub>50</sub> (18), and (b) decreasing receptor number by chemical inactivation resulted in a maximum increase in EC<sub>50</sub> to 2.4 nM, a value similar to the *K<sub>d</sub>* of GnRH for binding (23). Therefore the increase in EC<sub>50</sub> for GnRH to 24.1 nM (Table I) without any accompanying decrease in maximal inositol phosphate response, as exhibited by the [Ala<sup>102</sup>]GnRHR, must be due predominantly or entirely to decreased affinity.

Binding experiments were performed with a number of different iodinated peptide radioligands to determine directly the binding affinity of the [Ala<sup>102</sup>]GnRHR. While specific radioligand binding by transiently expressed wild-type GnRHR in COS-1 cells was easily measurable, binding by the mutant receptor was undetectable, consistent with decreased affinity for the radioligands.

To probe the possibility that Asn<sup>102</sup> was a contact residue for GnRH, dose-response curves for inositol phosphate production were performed using a number of GnRH agonist analogs differing at various positions on the peptide, (Table I and Fig. 1). The peptides [Trp<sup>2</sup>]GnRH, [His<sup>5</sup>,D-Tyr<sup>6</sup>]GnRH, [D-Ala<sup>6</sup>]GnRH,

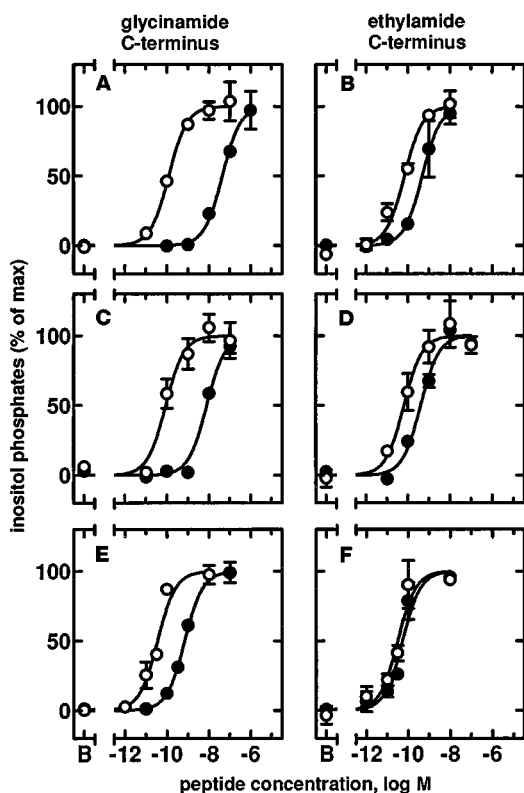


FIG. 1. Dose-response curves for inositol phosphate production stimulated by pairs of GnRH agonist analogs differing only at their C termini. Peptide numbering as in Table I is shown in parentheses. A, GnRH (peptide 1); B, [Pro<sup>9</sup>-NEt]GnRH (peptide 2); C, [D-Ala<sup>6</sup>]GnRH (peptide 4); D, [D-Ala<sup>6</sup>,Pro<sup>9</sup>-NEt]GnRH (peptide 5); E, [D-Trp<sup>6</sup>]GnRH (peptide 6); F, [D-Trp<sup>6</sup>,Pro<sup>9</sup>-NEt]GnRH (peptide 7). Open circles, human wild-type GnRHR; filled circles, [Ala<sup>102</sup>]GnRHR.

[D-Lys<sup>6</sup>]GnRH, [ $\gamma$ -lactam<sup>6,7</sup>]GnRH, [Gln<sup>8</sup>]GnRH, [Ala<sup>9</sup>]GnRH, and [His<sup>5</sup>,Trp<sup>7</sup>,Tyr<sup>8</sup>]GnRH (chicken II GnRH) all demonstrated potency losses between 94- and 750-fold in the mutant receptor compared with wild-type receptor (peptides 4 and 8–14, Table I). All of these analogs contain glycinamide at their C termini, as does the natural ligand GnRH, and their decrease in potency for the [Ala<sup>102</sup>]GnRHR was similar to that of the natural ligand. One peptide containing a C-terminal glycinamide, [D-Trp<sup>6</sup>]GnRH, showed only a mean 27-fold loss of potency in the mutant receptor compared with wild-type receptor (peptide 6, Table I). Analogs with amino acid substitutions at positions 1, 3, and 4 were not studied, either because of nonavailability of such peptides or on account of their very low affinities for the wild-type receptor. Very low affinity analogs were considered unsuitable for the purpose of delineating binding site contact residues because of the likelihood that they might bind in a different configuration from GnRH and its high affinity analogs.

In contrast, peptides in which the C-terminal glycinamide of GnRH is substituted with ethylamide, [Pro<sup>9</sup>-NEt]GnRH, [D-Ala<sup>6</sup>,Pro<sup>9</sup>-NEt]GnRH, [D-Trp<sup>6</sup>,Pro<sup>9</sup>-NEt]GnRH, and [D-Ser(Bu<sup>t</sup>)<sup>6</sup>,Pro<sup>9</sup>-NEt]GnRH (buserelin) showed only small increases in EC<sub>50</sub> between 2.5- and 11-fold (peptides 2, 5, 7, and 15; Table I). Thus the ethylamide-substituted peptides displayed less selectivity for the wild-type receptor than peptides with glycinamide C termini. Fig. 2 shows a graphical representation of these data (-fold potency loss plotted against log [EC<sub>50</sub>] for each analog) and reveals the clear delineation of the glycinamide versus ethylamide peptides in terms of potency reduction by the mutation.

Direct comparisons of three peptide pairs differing only at

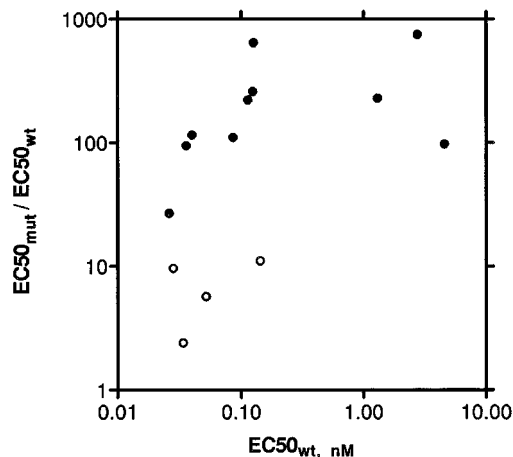


FIG. 2. Selectivity of GnRH analogs for wild type GnRHR over [Ala<sup>102</sup>]GnRHR. For each agonist, the potency loss for mutant versus wild-type receptor ( $EC_{50mut}/EC_{50wt}$ ) is plotted against  $EC_{50wt}$ . Filled circles, peptides with glycinamide C termini; Open circles, peptides with ethylamide C termini.

their C termini (peptides 1 and 2, 4 and 5, 6 and 7; Table I), revealed that in each pair, the glycinamide derivative was more dependent on Asn<sup>102</sup> for potency than its corresponding ethylamide derivative (Fig. 1). The C-terminal ethylamide substitution had little effect on the potency of these agonists for the wild-type receptor. However, when interacting with the [Ala<sup>102</sup>]GnRHR, the ethylamide-substituted peptides showed markedly higher potency than their glycinamide-containing derivatives. In these three peptide pairs, the C-terminal glycinamide moiety *per se* accounted for an 11–20-fold selectivity for wild-type GnRHR compared with [Ala<sup>102</sup>]GnRHR (Table I and Fig. 1).

GnRH-OH, which has a free carboxyl group at its C terminus, was 325-fold less potent than GnRH and exhibited a 157-fold selectivity between mutant and wild-type receptors, a value similar to that found for the glycinamide-containing analogs (Table I).

#### DISCUSSION

Systematic variation of the structure of a ligand as well as mutation of its receptor offers the possibility of identifying specific ligand-receptor contact points. In principle, removal of an interacting side chain of the receptor by mutating the residue to Ala, should produce a decrease in affinity that is similar in magnitude to, but nonadditive with, removal of the interacting group from the ligand. The ratio  $EC_{50mut}/EC_{50wt}$  is a measure of the loss of potency for an analog due to the receptor mutation. We demonstrate here that removal of the side chain of Asn<sup>102</sup> of the human GnRHR resulted in a 225-fold potency loss of GnRH for stimulation of inositol phosphate production. Thus for any particular GnRH analog, a potency loss equal to that for GnRH would indicate that the analog in question retains the same interaction with Asn<sup>102</sup> as does GnRH. The converse result, *i.e.*, a potency loss significantly less than that for GnRH, would suggest that the interaction of the peptide with Asn<sup>102</sup> is diminished or absent.

Eight GnRH analogs with substitutions at amino acids 2, 5, 6, 7, 8, or 9 exhibited potency losses of 94–750-fold for the [Ala<sup>102</sup>]GnRHR relative to the wild-type receptor (Table I). Since these potency losses are similar to the value of 225 for GnRH, these results argue against an interaction of Asn<sup>102</sup> with the side chains of residues at positions 2, 5, 6, 7, 8, and 9 in the peptide. Four of these peptides had glycine at position 6 as in GnRH, and four were “constrained” analogs with D-Ala<sup>6</sup>, D-Lys<sup>6</sup>, D-Tyr<sup>6</sup>, or  $\gamma$ -lactam<sup>6,7</sup> substitutions, which enhance

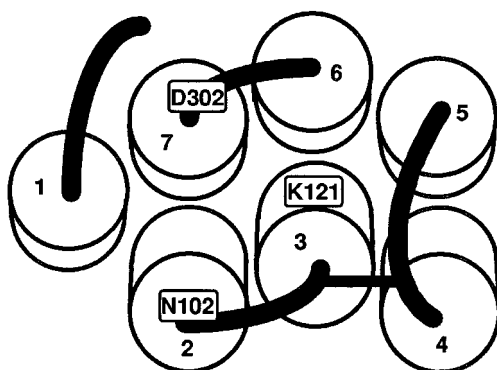


FIG. 3. Schematic representation of the GnRH receptor viewed from extracellular face. Numbered cylinders represent transmembrane helices, whereas solid lines represent extracellular interconnecting loops and N-terminal domain (truncated). Extracellular loops 1 and 2 are shown joined by a disulfide bridge. Residues Asn<sup>102</sup>, Lys<sup>121</sup>, and Asp<sup>302</sup> are implicated as ligand contact sites.

binding affinity and have been widely used in both agonist and antagonist GnRH analogs (11). It has been proposed that these substitutions stabilize the formation of a  $\beta$ -II type bend in the peptide, resulting in a preferred conformation for binding (24, 25). The "constrained" analogs were included in the series because we had previously found that these peptides showed a different pattern of behavior from Gly<sup>6</sup>-containing peptides with respect to their interaction with the mouse [Glu<sup>301</sup>]GnRHR (26). The present data show, however, that they are similar to the Gly<sup>6</sup>-containing peptides in that they display high selectivity for the wild-type receptor over the [Ala<sup>102</sup>]GnRHR.

In contrast, four analogs with an ethylamide substitution for glycylamide at the C terminus exhibited much smaller losses of potency, between 2.4- and 11-fold, for the mutant receptor, indicating that these peptides were all markedly less dependent for high potency on the presence of Asn<sup>102</sup>. The role of the ethylamide substitution *per se* was evaluated by comparing the potencies of three pairs of peptides, each differing only at the C terminus. Within each pair, the glycylamide derivative showed an 11–20-fold greater potency loss for the [Ala<sup>102</sup>]GnRHR than its corresponding ethylamide derivative.

[D-Trp<sup>6</sup>]GnRH was somewhat anomalous in that it exhibited only a 27-fold loss of potency for the [Ala<sup>102</sup>]GnRHR compared with wild-type GnRHR, despite the fact that it has a glycylamide C terminus (Fig. 1, Table I). This indicates that the D-Trp<sup>6</sup> substitution *per se* decreased the dependence on Asn<sup>102</sup> for binding, presumably as a result of an interaction of the D-Trp<sup>6</sup> side chain with another site on the receptor. However, its ethylamide derivative showed a loss of potency of only 2.4-fold, indicating that, similar to the other peptide pairs, the glycylamide moiety was independently responsible for an 11-fold selectivity of this peptide for the wild-type receptor over the [Ala<sup>102</sup>]GnRHR.

These results indicate that Asn<sup>102</sup> is critical in determining high potency for peptides with glycylamide C termini, while analogs with ethylamide C termini are less dependent on the presence of Asn<sup>102</sup>. The most direct interpretation of these data suggests that the Asn<sup>102</sup> side chain is a contact point for glycylamide-containing ligands and, more specifically, that Asn<sup>102</sup> is hydrogen bonded with the C-terminal amide group of GnRH. It should be noted that the ethylamide peptides do show some dependence on Asn<sup>102</sup> (although less than the glycylamide peptides), indicating that Asn<sup>102</sup> has an additional interaction with the ligand other than this proposed H-bonding with the C-terminal amide group. An alternative explanation, which cannot be excluded by the present data, is that the Asn<sup>102</sup> side chain plays a role in configuring the agonist binding pocket,

such that mutation to Ala severely restricts access of agonists with glycylamide, whereas it affects access less for agonists with the less bulky ethylamide C terminus.

The importance of the C terminus of GnRH for binding was demonstrated in early studies that showed that [des-Gly<sup>10</sup>,Pro<sup>9</sup>-NH<sub>2</sub>]GnRH, which lacks the C-terminal glycylamide, had very low potency (11). The finding that the addition of an ethyl moiety to the C terminus of the latter peptide, resulting in [Pro<sup>9</sup>-NET]GnRH, restored high potency indicates that the ethylamide peptides have gained an interaction, most likely a hydrophobic interaction, which compensates for the loss of the glycylamide. This explains why the ethylamide analogs retained potencies for the wild-type GnRHR comparable with their respective glycylamide analogs, despite the fact that the ethylamide C terminus is incapable of hydrogen bonding.

GnRH-OH, which has a carboxyl C terminus, was 325-fold less potent than GnRH for the wild-type GnRHR and showed a further 157-fold loss of potency for the [Ala<sup>102</sup>]GnRHR. The latter finding indicates that the C-terminal carboxyl group interacts with Asn<sup>102</sup> in a manner similar to a C-terminal amide group, consistent with a hydrogen bonding interaction. The low potency of this analog for both receptors indicates that there is some additional effect of the C-terminal carboxyl group, which has adverse consequences for binding. Two possible interactions suggest themselves. First, the C-terminal carboxyl could interact electrostatically with Arg<sup>8</sup> of GnRH-OH, which would be likely to have a marked effect on the conformation of the peptide. Alternatively, the low potency of GnRH-OH might be the result of the higher desolvation energy of the carboxyl *versus* the amide group in water.

We have previously demonstrated that Glu<sup>301</sup> in the mouse GnRHR is a specific determinant of the selectivity of the GnRHR for unconstrained GnRH analogs with Arg in position 8 of the peptide, suggesting an ionic interaction between Glu<sup>301</sup> and Arg<sup>8</sup> (26). Glu<sup>301</sup> (equivalent to Asp<sup>302</sup> in the human GnRHR) is situated near the top of transmembrane helix 7. Based on a survey of conserved residues in a large number of G-protein-coupled receptor sequences, as well as mutagenesis data, an arrangement of the transmembrane helices has been proposed in which helix 7 is believed to lie adjacent to helix 2 (27, 28). This arrangement of the helices is supported by experimental evidence in the mouse GnRHR, in which an analysis of the effects of reciprocal mutations of Asn<sup>87</sup> (helix 2) and Asp<sup>318</sup> (helix 7) has suggested that these residues are in proximity (29). Thus Asn<sup>102</sup> and Asp<sup>302</sup> are likely to be in close proximity in the three-dimensional structure of the GnRHR and may define a binding pocket accommodating Arg<sup>8</sup> and the C terminus of the ligand, as shown schematically in Fig. 3. This site is also close in space to Lys<sup>121</sup>, located in the extracellular third of transmembrane helix 3, which has been shown to be a likely contact site for agonists but not antagonists (23).

In summary, we have shown that Asn<sup>102</sup> of the GnRHR is a critical determinant of potency for GnRH and analogs containing glycylamide at the C terminus. The results are consistent with a direct interaction between the side chain of Asn<sup>102</sup> and the C terminus of the ligand. Alternatively, the effects of mutating Asn<sup>102</sup> may be mediated indirectly and reflect a role for this residue in maintaining the conformation of the ligand binding pocket.

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**Asn<sup>102</sup> of the Gonadotropin-releasing Hormone Receptor Is a Critical Determinant of Potency for Agonists Containing C-terminal Glycinamide**

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