

EVOLUTION OF CONSTRAINED GnRH LIGAND CONFORMATION AND RECEPTOR SELECTIVITY

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Gonadotropin Releasing-Hormone (GnRH) is the central regulator of reproduction in vertebrates. GnRHs have recently been identified in protochordates and retain the conserved NH₂- and COOH-terminal domains involved in receptor binding and activation. GnRHs of the jawed vertebrates have a central achiral amino acid (glycine) which favours a type-II'- β -turn such that the NH₂- and COOH-terminal domains are closely apposed in binding the GnRH receptor. However, protochordate GnRHs have a chiral amino acid in this position suggesting that they bind their receptors in a more extended form. We demonstrate here that a protochordate GnRH receptor does not distinguish GnRHs with achiral or chiral amino acids while GnRH receptors of jawed vertebrates are highly selective for GnRHs with the central achiral glycine. The poor activity of the protochordate GnRH was increased >10-fold at vertebrate receptors by replacement of the chiral amino acid with glycine or a D-amino acid which favour the type-II'- β -turn. Structural analysis of the GnRHs using ion-mobility-mass-spectrometry and molecular modelling showed a greater propensity for a type-II'- β -turn in GnRHs with glycine or a D-amino acid which correlates with binding affinity at vertebrate receptors. These findings indicate that the substitution of glycine for a chiral amino acid in GnRH during evolution allows a more constrained conformation for receptor binding, and that this subtle single amino acid substitution in a site remote from the ligand functional domains has marked effects on its structure and activity.

In vertebrates, Gonadotropin Releasing-Hormone (GnRH) is synthesised in hypothalamic neurones and conducted a few millimetres in the hypophyseal portal system to the anterior pituitary where it binds to high affinity receptors in gonadotropes to stimulate the release of gonadotropins (1). The gonadotropins in turn stimulate hormone and gamete production by the testes and ovaries. GnRHs have also been isolated from protochordate species (2,3) and are thought to be secreted from neurones to directly regulate the gonads (2-4) in these representatives of vertebrate progenitors. GnRHs and GnRH receptors have also been found to directly affect vertebrate gonadal function (5) possibly reflecting the earliest role of GnRH as exemplified in protochordates (6,7). It appears, therefore, that GnRHs have an ancient evolutionary role as regulators of reproduction, first through direct neural delivery to the gonads and later as hypothalamic neuroendocrine regulators of the gonads indirectly through gonadal stimulation by gonadotropins.

To date, 13 structural variants of the GnRH decapeptide have been identified in vertebrates (8), 9 from protochordates which are vertebrate progenitors (8,9) and a twelve amino acid homolog from an octopus species (10) (Fig. 1). All of the GnRHs are characterised by the conservation of the NH₂-terminal residues (pGlu-His-Trp-Ser) and the COOH terminal residues (Pro-Gly-NH₂) with the exception of two conservative substitutions (Fig. 1). In cartilaginous and bony fish, amphibians, reptiles, birds and mammals, all of the GnRHs are further characterised by the presence of a glycine residue in position six (Fig. 1). Being archiral, the glycine

residue allows the peptide to assume a type-II'- β -turn conformation which is essential for high binding affinity and biological activity in mammals (1,8,11,12). Substitution of glycine with any other L-amino acid decreases biological activity as the type-II'- β -turn conformation is less favoured (1,8,11,12). In contrast, substitution of glycine with D-amino acids in position six constrains the peptide to the type-II'- β -turn conformation and increases binding affinity and biological activity in mammals, birds, amphibians and jawed fish species (1,6-8,11,12).

The GnRHs in the ancient jawless lamprey and protochordate species (with the exception of Ciona VI) are all characterised by the presence of chiral amino acids in position six (Fig. 1). This feature is likely to limit the formation of a type-II'- β -turn conformation and these GnRHs would be expected to have correspondingly low binding affinities and biological activities at GnRH receptors of higher vertebrates (1,6,7,12).

We therefore hypothesized that early in evolution GnRH interacted with the GnRH receptor in a more relaxed (linear) conformation. Coincident with the evolution of the jawed fish, structural changes in GnRH and its receptor required the peptide to interact in the folded type-II'- β -turn conformation. To investigate this notion, we have synthesised and studied the structure and biological activity of three GnRHs from a tunicate protochordate (*Ciona intestinalis*), at catfish, chicken, human and protochordate GnRH receptors. We demonstrate that these protochordate GnRHs have low biological activity at jawed vertebrate GnRH receptors but that high biological activity is generated when the natural chiral amino acid in position six is replaced by the achiral glycine which is characteristic of jawed vertebrate GnRHs. We also studied the structure of these peptides employing a combination of Ion Mobility Mass Spectrometry (IM-MS) and molecular modelling, and demonstrated a correlation of the biological activity at vertebrate receptors with the ability of the peptides to assume the folded more compact conformation. These findings reveal a co-evolution of GnRH and its cognate receptor in which the receptors of early evolved organisms bind an extended structure of GnRH while the receptors of higher organisms require a folded type-II'- β -turn conformation of

GnRH in which the NH₂- and COOH-termini are in close apposition when bound to the receptor.

EXPERIMENTAL PROCEDURES

Peptides Ciona I, II and III GnRH and Gly⁶-Ciona I GnRH and D-Ala⁶-Ciona I GnRH were synthesized by conventional solid phase methodology and purified by HPLC to >98% purity. Mammalian GnRH was from Peninsula Laboratories (Bachem (UK) Ltd., Merseyside).

Cell culture and transient transfection Plasmid DNA for transient transfection was prepared using Maxi-Prep columns (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions. COS-7 and HEK 293 cells were cultured as previously described for COS-1 cells (12,13) and transiently transfected with the human (14), chicken (15) and catfish (16) GnRH receptors as well as the recently-cloned Ciona receptors (17)⁷ using electroporation. These are Ciona A and Ciona B GnRH receptors previously referred to as Ci-GnRHR1 and Ci-GnRHR2.

Receptor binding assays Whole cell receptor binding assays used the ¹²⁵I-[His⁵,D-Tyr⁶]GnRH analog for the vertebrate receptors and ¹²⁵I-GnRH II for the Ciona receptor (13). Transiently transfected COS-7 cells in 12-well culture plates were washed once with ice-cold HEPES/DMEM/10% fetal calf serum and incubated for 5h on ice in the same medium with with 10⁶ cpm/well radio-labelled GnRH analog and varying concentrations of unlabeled GnRH peptides (13). Cell monolayers were then rapidly washed twice in ice-cold PBS, solubilized in 0.1M NaOH and the lysate radioactivity counted. Non-specific binding (consistently less than 10% of total binding) was determined using vector-transfected (pcDNA1/amp) COS-7 cells and was subtracted from total binding to give specific binding. Assays were conducted in triplicate and repeated in three separate experiments.

Total inositol phosphate assays GnRH stimulation of total inositol phosphate production was assayed as previously described (18,19). Briefly, transiently transfected COS-7 cells were incubated with inositol-free DMEM containing 1% dialyzed heat-inactivated FCS and 0.5 μ Ci/well myo-

[³H]inositol (Amersham Pharmacia Biotech, Piscataway, NJ) for 48h. Medium was removed, and the cells were washed with 1ml buffer (140mM NaCl, 20mM HEPES, 4mM KCl, 8mM glucose 1mM MgCl₂, 1mM CaCl₂, and 1mg/ml BSA) containing 10mM LiCl and incubated for 1h at 37C in 0.5 ml buffer containing 10mM LiCl and GnRH agonist at the indicated concentration. Reactions were terminated by the removal of agonist and the addition of 1ml ice-cold 10mM formic acid, which was incubated for 30 min at 4C. Total [³H]-inositol phosphates were separated from the formic acid cell extracts on AG-X8 anion exchange resin (Bio-Rad Laboratories, Hercules, CA) and eluted with a 1M ammonium formate/0.1M formic acid solution. The associated radioactivity was determined by liquid scintillation counting. Assays were conducted in triplicate and repeated in three separate experiments.

Ion Mobility-Mass Spectrometry The gas-phase collision cross-sections of mammalian GnRH, Ciona I GnRH and its two substituted derivatives were determined using an Ion Mobility Mass Spectrometer, as previously described (20). Briefly, ions created by electrospray ionisation are injected into a temperature regulated drift cell pressurised with helium to 5 torr. The ions drift under the influence of a weak electrostatic field and are retarded by collisions with the buffer gas. On exiting the cell, ions are selected by a quadrupole mass filter and their arrival time distributions are recorded. Arrival times are collected at several drift voltages, and the mobility (K) of the ion is obtained from the gradient of a plot of arrival time versus the pressure of helium divided by the drift voltage. This mobility is used to determine the experimental collision cross-section of the ion (σ) according to the equation,

$$K = [(3e)/(16N)][(2\pi)/(\mu k_B T)]^{1/2} [1/\sigma], \text{ as described (21).}$$

Molecular structures of GnRH peptides Analysis of the structures of mammalian GnRH, Ciona I GnRH and two substituted Ciona I variants was conducted using molecular mechanics. All calculations were performed using the ff99 AMBER force field, (22) employing the Amber7 suite of programs to refine the energy of the peptide models. Residues for the N-terminal

pyrolysed glutamic acid and the amidated glycine present at the C-terminus generated using the RESP procedure (23) and the ANTECHAMBER facility within the Amber 7 suite of programs (<http://amber.scripps.edu>). IM-MS data was obtained for these peptides both as singly ([M+H]⁺) and doubly ([M+2H]²⁺) protonated ions. From gas phase basicities (24) it is most probable that Arg⁸ in mammalian GnRH will be protonated in [M+H]⁺ and that the [M+2H]²⁺ ion will contain protonated His². In the protochordate Ciona I peptides, Arg⁸ is substituted by Ser⁸ and it is extremely likely that their [M+H]⁺ ions contain protonated histidine. The protonation state of His² is variable in physiological conditions, and so calculations were performed with both protonated and unprotonated histidine for the Ciona I peptides, and for the singly and doubly protonated form of mammalian GnRH. At most physiological pHs, Arg⁸ of mammalian GnRH will be protonated, hence calculations on the neutral species have not been performed for this peptide. The Ciona I [M+2H]²⁺ ions probably arise due to protonation of the amide Gly¹⁰, however since we cannot determine the biological significance of this chemical form, and due to some ambiguity in assigning this site of charge, the doubly protonated peptides were not investigated further in this study. For each of these 8 chemically distinct peptides, 300 candidate gas-phase minimised structures were generated using a simulated annealing approach. An initial structure was subjected to dynamics at 800K for 30ns and then cooled in a stepwise fashion to 0K prior to an energy minimisation. This minimised structure was then used as the seed for the next cycle. The two lowest energy structures for each species were then subjected to 1ns of molecular mechanics at 300K, for the [M+H]⁺ ions. This was performed *in vacuo* and employing the Born Solvent distance-dependent dielectric model to simulate their dynamic structure in an aqueous environment (25). For the neutral Ciona I peptides, these extended dynamics calculations were only performed with the use of the solvent model. This procedure generates 1000 co-ordinate files for each 1ns, i.e. co-ordinates are saved every ps, with the potential energy surface being sampled every fs. No weighting was given to any of the amino acids, and translational energies are removed every ps. Each molecular mechanics data set was analysed

and conformational variables, such as the N-C termini distance and overall peptide flexibility were compared to those found for mammalian GnRH examined under identical conditions. Collision cross-sections for each low energy structure obtained via the simulated annealing procedure were calculated using the projection approximation (26). This was also employed to elucidate conformational changes occurring to these peptides during molecular dynamics.

RESULTS

Receptor binding All three Ciona GnRHs had low affinity for the human receptor ($IC_{50} > 1 \mu M$) in comparison to mammalian GnRH (7.5 nM) (Fig. 2A, Table I). Similar results were found in binding of these peptides to the chicken (Fig. 2D) and catfish (data not shown) GnRH receptors. In contrast Ciona I and Ciona II had low affinities (918 nM and 988 nM) at the Ciona A receptor which were similar to that of mammalian GnRH (1390 nM) (Fig. 2B). Ciona II had a considerably lower affinity (5600 nM) and may be the cognate ligand for a second Ciona GnRH receptor (17) which expressed poorly and therefore did not allow binding studies (data not shown). As Ciona I was the most active of the Ciona peptides at the human receptor, this was selected for studies to determine if substitution of the chiral amino acid in position six would increase activity at the human receptor. Replacement of Ala⁶ with the achiral Gly⁶ or with the type-II'- β -turn-constraining D-Ala⁶ resulted in a marked increase (36- to 46-fold) in binding affinity (Fig. 2C, Table I). Similar results were obtained for the chicken (Fig. 2D) and catfish (data not shown) GnRH receptors.

Inositol phosphate production The three Ciona peptides were at least three orders of magnitude less potent than mammalian GnRH at stimulating inositol phosphate production (Fig. 3, Table I). Their relative activities paralleled that of their binding affinities. Substitution of Ala⁶ with Gly⁶ or D-Ala⁶ increased the potency more than 100-fold.

Ion Mobility-Mass Spectrometry and molecular modelling of GnRH peptides Experimental cross-sections measured for the $[M+H]^+$ ions of

mammalian GnRH, Ciona I and Ciona II GnRH analogs are given in Table II. Agreement between the measured and calculated values is very good. The gas phase cross-section of mammalian GnRH is smaller than that obtained for the Ciona I GnRH and substitution of L-Ala⁶ with D-Ala⁶ or Gly⁶, reduces the cross-section to now approach that of mammalian GnRH. Figure 4 shows representative low energy structures obtained from the *in vacuo* simulations of the peptide ions. The general conformation of all of the peptides is comparable, as might be expected based on their sequence homologies. All form compact structures with a hairpin shape to the polypeptide backbone, which is sustained through non-covalent interactions across this structure. The radius is larger for the Ciona I form than that found for the Gly⁶ or D-Ala⁶ substituted Ciona I (Fig. 4) in agreement with the IM-MS cross-section data (Table II). Cross-sections obtained from the geometry calculations with unprotonated histidine are also included.

By performing molecular dynamics on these low energy structures, the conformational space available to them is sampled, thus probing the stability of interactions formed. This was conducted both *in vacuo* and also with the application of a solvent model. Conformations obtained with the Born Solvent model are presumed to be more representative of biologically active conformations, at least prior to receptor binding. Several criteria were examined in the room temperature dynamics and our findings are summarised below and in Table III.

N- to C-Terminal Distance The proposal that GnRH binds the GnRH mammalian receptor in a type-II'- β -turn conformation, suggests non-covalent interaction's which position the N- and C-terminal residues closely apposed in space. The low energy gas-phase structures determined here fulfil this criterion (Fig. 4). Over the 1ns dynamics runs, the persistence of this interaction was explored by plotting the distance between the alpha carbons of the N-terminal pyrolysed glutamic acid and the C-terminal glycine amide. The *in vacuo* simulations for mammalian GnRH and Ciona I GnRH and analogs reveal a continual N-C terminal proximity, which bears out the analysis made above on the low energy conformation (Table III). It is apparent that the distance between the alpha carbons decreases as

residue 6 is replaced by D-Ala or achiral glycine. With application of the Born-Solvent distance dielectric function, Ciona I GnRH exhibits a somewhat larger average N-C terminal *variation* than D-Ala⁶-Ciona and significantly larger than for Gly⁶-Ciona I (Table III). Mammalian GnRH displays a more rigid conformation, with less deviation from the average N-C distance than the Ciona I peptides. However all of the forms of GnRH experience an opening of the peptide backbone within the higher dielectric environment, resulting in a larger average N-C terminal distance as seen in representative structures obtained from the dynamics runs under Born Solvent conditions (Fig. 5).

Trp³-Leu⁷ distances Whilst it is clear that the GnRH peptides exhibit considerable conformational flexibility their dominant structural feature is a type-II'- β -turn between residues 5 and 8. The increase in receptor binding affinity when the achiral Gly or D-Ala is substituted for the chiral L-Ala in Ciona I GnRH is attributed to a release in steric hindrance allowing for a tighter hinge to the peptide backbone. We have examined the distance between Trp³ and Leu⁷, as a possible indicator of hinge mobility. For Ciona I and the substituted analogs, the average distance between these residues is remarkably similar (Table III). It is however, approximately 8-16% more than the average value found for the mammalian GnRH, modelled under identical conditions. In the gas-phase simulations this effect is most marked. This suggests that the primary sequence, and specifically the Ser⁸ to Arg⁸ substitution, has a large effect on retaining the rigidity of this type-II'- β -turn.

Conformational Flexibility Two methods were employed to assess conformational flexibility. The first examined the rotationally averaged collision cross-section of each peptide conformation during the course of the 1ns dynamics run. The second, more conventional approach, examined the RMS deviation of the amino acids from their positions in an averaged structure. Both methods gave comparable results. The *in vacuo* data essentially confirms the findings discussed above. Over the course of the dynamics the peptides retain their compact conformations, and significantly, the size ordering remains L-Ala⁶ > D-Ala⁶ > Gly⁶. With

the application of the solvent model, collision cross-sections increase for all the GnRH peptides, reflecting looser, and at times, elongated structures. The overall conformational flexibility of Ciona I GnRH was greater than that of the mammalian GnRH. Over the course of 1ns of molecular dynamics at 300K Ciona I GnRH exhibited more frequent conformational shifts than Gly⁶ Ciona I and mammalian GnRH (Fig. 6). In the dynamics runs, the average cross-section of Ciona I [M] (Fig. 6A) was $275.9 \pm 12.2 \text{ \AA}^2$, and Gly⁶ Ciona I [M] (Fig. 6B) was $266.52 \pm 5.2 \text{ \AA}^2$ (Table III). These trends are also seen in the [M+H]⁺ protonated histidine forms. The D-Ala⁶ and Gly⁶ forms of Ciona I and mammalian GnRH retain a centrally configured loop in the solvent calculations (Fig. 5B and C). Comparison with the data from mammalian GnRH (Fig. 5C) shows that this form of the peptide also appears somewhat stabilised during dynamics although it also exhibits an increase in the N-C terminal distance. The overall cross-section of mammalian GnRH is larger due to the Arg⁸. Interestingly this bulky side chain of Arg⁸ is rather flexible which supports data obtained from both the mouse (15) and the human (16) receptors that demonstrate Arg⁸ is integral to binding via an acidic residue in extracellular loop 3 (15,16). The jump in cross-section in the earlier part of these dynamics measurements is expected, as the temperature of the low energy starting conformers is brought to thermal equilibration.

Although no attempt was made to model the interaction of the peptide with the receptor, it is apparent that the configurations of the isolated peptides can be related to the biological activity. The correlation between the experimentally measured cross-sections (Table II) and the stimulation of inositol phosphate data (Table I) is very good. The small tightly configured mammalian GnRH exhibits the strongest activity at the receptor whereas the larger 'looser' Ciona I L-Ala analog, is the weakest binder. The D-Ala⁶- and Gly⁶-Ciona I peptides are intermediate in cross-sections and binding affinity. This, coupled with the fact that all the gas-phase structures have proximal N-C termini, suggests that the gas-phase conformations are comparable to the receptor bound form.

DISCUSSION

The importance of having the achiral glycine in position 6 of mammalian GnRH for biological activity was demonstrated in empirical studies three decades ago (1,11,27). Subsequent studies using NH₂- and COOH-terminally directed antibodies, fluorescence spectroscopy, molecular modelling and NMR suggested that Gly⁶ was essential to allow GnRH to assume a type-II'-β-turn conformation (1,8,11). Although GnRH receptors had not been cloned when this concept was proposed, these findings together with numerous structure-activity studies indicated that mammalian GnRH interacted with its cognate receptor in this type-II'-β-turn conformation through the amino (pGlu-His-Trp-Ser) and carboxyl (Arg-Pro-Gly.NH₂) terminal domains (1,6-8). The subsequent elucidation of GnRH structural variants in vertebrates and protochordates reveals the conservation of these domains over more than 500 million years of evolution thus supporting this conclusion (Fig. 1). Mutation of amino acids in cloned human and rat GnRH receptors has provided direct evidence for the interaction of individual amino acids in these two ligand domains with cognate receptor residues (pGlu¹ with N²¹²; His² with K¹²¹ and D⁹⁸; Trp³ with W²⁸⁰; Arg⁸ with D³⁰²; and Gly.NH₂¹⁰ with N¹⁰²) (8,18,19,28-30). The receptor residues which bind the conserved pGlu, His, Trp, Ser and Gly.NH₂ of Ciona GnRHs are all conserved in the Ciona receptors as in fish, amphibian and chicken receptors (8) with the exception of N²¹² which is a tyrosine in the Ciona A receptor.

In view of the stringent requirement of jawed vertebrate GnRH receptors for a glycine residue in position six to allow presentation of the NH₂- and COOH-terminal domains in a folded conformation to the receptor, the presence of chiral amino acids in position six in jawless fish and protochordate GnRHs was unexpected. This finding suggests that the receptors of these species are able to interact with GnRH in an extended conformation. The similar low binding affinities (~1μM) of mammalian, Ciona I and Ciona III GnRHs at the Ciona A receptor supports this interpretation. The ability of the endogenous protochordate GnRHs and mammalian GnRH to stimulate spawning in protochordate species (2,3,9) also indicates that protochordate GnRH receptors do not distinguish mammalian and protochordate GnRHs. GnRHs with an achiral

amino acid in position six were similarly active in here mollusc species (31,32). Interestingly, one of the protochordate GnRHs (Ciona VI) has Gly⁶ and is active in stimulating spawning in Ciona intestinalis (9). This finding supports our data demonstrating that the Ciona A GnRH receptor binds GnRHs with chiral (Ciona I and III) and achiral (mammalian GnRH) amino acids in position six equally well. This is consistent with our interpretations as forms with chiral and achiral amino acids in position six can both assume a relaxed linear extended conformation. In contrast, we have shown here that the types of GnRH with chiral amino acids in position six have very low binding affinity at mammalian, avian and teleostean GnRH receptors.

Our molecular modelling and IM-MS studies demonstrate a correlation between the propensity for type-II'-β-turn conformation of GnRHs and their biological activity at the vertebrate receptors. Thus Ciona I GnRH assumes a looser and hence less configured structure than mammalian GnRH and has poor binding affinity at vertebrate receptors. By comparing the collision cross-sections from experimental and calculated structures it is apparent that L-Ala at position six induces steric hindrance to the formation of the more compact folded structure. This is supported by the larger Trp³-Leu⁷ distances found with Ciona I than for the Gly⁶ or D-Ala⁶ forms *in vacuo* and associated larger IM-MS collision cross-sections (Table II). We have previously investigated the gas-phase conformations of D-Trp substituted mammalian GnRH, by both experiment and calculation (33). Here we find that the D-Trp form adopts a much more compact geometry, with a type-II'-β-turn, whereas the L-Trp variant is more extended due to steric effects caused by the bulky side chain in the naturally occurring form. The solvent calculations we present here on the Ciona I variants show these effects more dramatically. L-Ala at position six allows the backbone to open out in the course of the dynamics simulations, producing a much more elongated and less compact form of the peptide. In contrast the D-Ala⁶ and Gly⁶ substituted Ciona I have more compact structure throughout the solvent dynamics. These findings concur with NMR studies on mammalian GnRH and D-Trp⁶-GnRH (34), but NMR is unable to distinguish structural differences between GnRHs without D-amino

acids in position six. The extensive molecular modelling of Guarnieri and Weinstein (35) revealed that the conformational preference for a type-II'- β -turn in the backbone of mammalian GnRH is significantly diminished by an Arg⁸ \rightarrow Lys⁸ substitution. Our gas phase results also indicate the functional importance of Arg in the mammalian form, where its guanidinium side chain is flexible and forms favourable interactions across the polypeptide backbone, which assists the type-II'- β -turn around glycine. Glycine residues are often found at turning points in protein structures, as the lack of side chain enables a tight turn to be made, but such turns are only successful if additional non-covalent interactions are present, as promoted here by Arg. This combination of Gly⁶ and Arg⁸ in mammalian GnRH produces a peptide configured with high affinity for the mammalian receptor. Substitution with D-Ala⁶ or Gly⁶ in Ciona I increases the affinity for the human receptor, since the peptide can now form a tighter turn. However the presence of Ser in position eight rather than Arg, precludes the very high affinity exhibited by the mammalian form at the mammalian receptor, due in part to the additional stabilisation of the compact structure by Arg, and the interaction of Arg with an acidic residue in extracellular loop 3 which contributes to configuration of the ligand at mammalian receptors (18,19).

When the chiral amino acid (Ala) in position six of Ciona I GnRH was substituted with the achiral glycine or with D-Ala which enhance the type-II'- β -turn conformation there was a marked increase in the binding affinity of the peptides at the vertebrate GnRH receptors. Moreover, the gas-phase studies indicated that these peptides assume a more compact configuration whilst in solvent calculations they become more flexible. Thus, there is a correlation between biological activity and the tighter conformation of the peptides at the vertebrate receptors whereas this situation does not pertain at the protochordate receptor.

These studies suggest that the early-evolved GnRH receptors of protochordates (and probably jawless fish) bind GnRH in a more extended configuration and that the subsequent evolution of the receptors in jawed vertebrates required a more compact configuration of GnRH

for binding. The conservation of the NH₂- and COOH-terminal domains of GnRHs in invertebrates and vertebrates (protochordates, jawless fish, jawed fish, amphibians, birds and mammals) nevertheless indicates that these domains are functionally important for binding and activating the GnRH receptor. As mentioned earlier most of the binding sites for the NH₂- and COOH-terminal GnRH residues in the human and vertebrate receptors are present in the tunicate GnRH receptor. However, the non-requirement for an achiral amino acid in position six in protochordates and jawless fish, but the requirement for an achiral Gly in higher vertebrates indicates that, although the NH₂- and COOH-domains of GnRH interact through the same or similar sites in the receptor, these are more closely positioned in the higher vertebrates (which require the folded conformation) than in protochordates and jawless fish (which bind less configured forms of the peptide equally well and with lower affinity). It is possible, therefore, that the protochordate GnRH receptor is also less compact due to fewer transmembrane domain interactions. A major transmembrane domain interaction in GPCRs is between Asp^{2.50} in TM2 and Asn^{7.50} in TM7. These are reciprocally mutated to Asn and Asp in the human GnRH receptor and these residues are critical for receptor function (36). In the Ciona GnRH receptors Asn is present in TM2 but His replaces Asp in TM7. In addition to this TM interaction we recently identified Met²²⁷ (TM5), Phe²⁷² (TM6), Phe²⁷⁶ (TM6) and Ile³²² (TM7) as interacting residues in the human GnRH receptor which alter ligand affinity and selectivity when mutated (37). Ph²⁷⁶ is conserved between the tunicate and human GnRH receptors. Met¹³² is Val, Met²²⁷ is Thr, Phe²⁷² is Ile, and Ile³²² is Val in the Ciona A receptor. These residue differences may therefore contribute to reduced ligand affinity and selectivity in the Ciona A receptor and will be the subject of future studies.

In addition to its direct activation of the gonads in Ciona, GnRH appears to serve a role as a pheromone in another protochordate, *Saccoglossus* (38), and in a mollusc (*Chiton*) (31). Tunicate spawning is precisely correlated with light cycles and light appears to stimulate GnRH secretion (39) which is secreted directly from nerve endings onto gonadal cells. In vertebrates,

however, GnRH has been co-opted to serve a more complex neuroendocrine role where external environmental factors (*e.g.* light) stimulate secretion of GnRH into portal vessels to stimulate secretion of pituitary gonadotropins and ultimately the gonads. The dilution of GnRH in the portal vessels may thus have driven the evolution of the more compact GnRH with higher affinity binding. However, some of the earlier-evolved functions seen in protochordates appear to have been retained in vertebrates as GnRH and GnRH receptors are present in the gonads of fish, amphibians and mammals (5-7) suggesting direct effects on gonads (40).

It is therefore evident that the GnRH structure existed very early in evolution and was co-opted in diverse ways to regulate reproduction. During at least 600 million years of evolution the NH₂- and COOH-termini of GnRH have been conserved as functional domains for binding and activating cognate receptors to accomplish these functions. However, about 400 million years ago a single substitution of the chiral amino acid in

position six of GnRH in jawless fish by the achiral glycine facilitated a type-II'- β -turn conformation of GnRH to allow spatially close interaction of these functional domains of GnRH with its receptor in contrast to the interaction of more extended GnRH structures with receptors in earlier-evolved species. This notion was supported by studies on receptor binding affinities and IM-MS molecular modelling conformations of GnRHs with Ala, Gly and D-Ala in position six which showed a close correlation between binding affinity at vertebrate GnRH receptors and their propensity to form a type-II'- β -turn conformation. Thus an apparently insignificant substitution of a single amino acid at a site remote from the binding and activation domains of GnRH can have a major effect on the conformation of the ligand and affect its interaction with the receptor binding sites. These findings emphasize the importance of subtle changes in three dimensional structural evolution of a peptide ligand which is likely to also pertain to other peptide ligands and their receptors.

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FOOTNOTES

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⁷ During revision of this manuscript Tello *et. al.* (Tunicate GnRH peptides selectively activate *Ciona intestinalis* GnRH receptors and the green monkey type II GnRH receptor. *Endocrinology*. 2005 Jun 16; [Epub ahead of print]) reported the cloning of these two receptors and two additional receptors which are selective for the various tunicate GnRHs.

FIGURE LEGENDS

Fig. 1. Primary amino acid sequences of naturally occurring decapeptide gonadotropin-releasing hormone (GnRH) structural variants spanning approximately 600 million years of evolution. The boxed regions show the conserved NH₂- and COOH- terminal residues which are involved in receptor binding and activation. The GnRHs are named according to the species in which they were first discovered and they may be represented in more than one species. For example, mammalian GnRH is widely conserved in amphibians and primitive bony fish, and chicken GnRH II is present in most vertebrate species, including man. An octopus GnRH and an additional Ciona GnRH comprising 12 and 16 amino acids respectively but retaining the conserved NH₂- and COOH-terminal domains are not shown.

Fig. 2. Binding of mammalian, Ciona and position six substituted Ciona I GnRHs to human, Ciona A and chicken GnRH receptors. COS-7 cells were transfected with human, Ciona A and chicken GnRH receptors, incubated with radio-labeled GnRH analogs and increasing doses of unlabeled GnRHs and analogs in triplicate, washed in PBS at 4°C and the bound radioactivity determined as described in methods. Points are mean±S.E. (within symbol size). Similar results to C and D were obtained with the catfish receptor (not shown). A and B, *Filled square*, mammalian GnRH; *filled triangle*, Ciona I GnRH; *open triangle*, Ciona II GnRH; *open circle*, Ciona III GnRH. C and D, *Filled square*, mammalian GnRH; *filled triangle*, Ciona I GnRH; *filled circle*, Gly⁶-Ciona I GnRH; *open square*, D-Ala⁶-Ciona I GnRH.

Fig. 3. Stimulation of inositol phosphate production by mammalian and Ciona GnRHs, and position six substituted analogues at the human GnRH receptor. COS-7 cells were transfected with the human GnRH receptor, incubated with myo-[³H]inositol and then GnRH analogs at increasing concentrations in the presence of LiCl, and total [³H]inositol phosphates determined as described in methods. Points are mean±S.E. of triplicates. *Filled square*, mammalian GnRH; *filled triangle*, Ciona I GnRH; *open triangle*, Ciona II GnRH; *open circle*, Ciona III GnRH; *filled circle*, Gly⁶ Ciona I GnRH; *open square*, D-Ala⁶ Ciona I GnRH.

Fig. 4. Ribbon representations of peptide structures. The peptide backbone for the [M+H]⁺ ions of (a) Ciona I, (b) Gly⁶ Ciona I, (c) D-Ala⁶ Ciona I, and (d) Mammalian GnRH are shown. Each is a snapshot from *in vacuo* dynamics calculations. The amidated N-terminal glycine is shown in each, as is residue six and the proton carrying residue. As described in text and Table III the cross peptide radius for Ciona I peptide is larger than for the other peptides (b, c, d) which possesses a tighter turn around residues 5 and 6. In all of these peptides the proton carrying group (histidine for the Ciona peptides and arginine for mammalian GnRH) ‘caps’ the polypeptide ring, thereby providing points for non-covalent interactions which stabilize these compact geometries of gas-phase ions.

Fig. 5. Ribbon representations of peptide structures. The peptide backbone of: (a) Ciona I, (b) Gly⁶ Ciona I, and (c) Mammalian GnRH are shown. The Ciona I peptides contain protonated His² and Mammalian GnRH has Arg⁸ and His² both protonated. Each is a snapshot from dynamics performed with the Born-Solvent model, with the N- and C-terminal residues represented by CPK structure. The distance between the alpha carbons of the terminal residues in each snapshot is marked. The D-Ala⁶-Ciona I GnRH structure was similar to Gly⁶-Ciona I (not shown).

Fig. 6. Cross-section measurements obtained from Born-Solvent model simulations for (a) Ciona I [M], (b) Gly⁶-Ciona I [M], (c) mammalian GnRH [M+H]⁺. The dynamics runs were performed for 1ns at 300K. The data shown is obtained by determining the collision cross-section of the structures output from these calculations every 1ps using the projection approximation (26). The initial spike observed here, in each plot, is an artifact, attributable to the effect of rapidly heating the peptide up to 300K from its 0K low energy structure. This data corresponds to the averaged values shown in Table III, and nicely

demonstrates the conformational flexibility of Ciona I compared to mammalian GnRH and to the Gly⁶ substituted form.

Table I
Receptor binding and inositol phosphate production by mammalian and Ciona GnRHs and analogues at the human GnRH receptor expressed in COS-7 cells.

Peptide	Receptor Binding IC ₅₀ (nM)		Inositol Phosphate Production ED ₅₀ (nM)	
mammalian GnRH	7.5±2.9	1.0 ^a	0.3±0.1	1.0 ^a
Ciona I GnRH	15,533±2,458	0.0005	4130±2016.3	0.00007
Ciona II GnRH	42,400±16,627	0.0002	24,467±6,816	0.00001
Ciona III GnRH	>100,000	<0.001	>100,000	<0.001
Gly ⁶ Ciona I GnRH	410±96.5	0.018	15.1±24.1	0.02
D-Ala ⁶ Ciona I GnRH	330.3±107	0.023	36.9±20.5	0.008

^aActivity relative to mammalian GnRH. Data are the mean±S.E. of three experiments done in triplicate.

Table II

Collisions cross-sections for the $[M+H]^+$ GnRH peptide ions measured experimentally and those obtained from the low energy candidate conformations. Values in parentheses are the range of cross-sections observed for the lowest 10 % of structures. Calculated collision cross-sections for the neutral peptides are listed in the third column.

GnRH	$[M+H]^+$ (\AA^2)		$[M]$ (\AA^2)
	Experimental	Calculated	Calculated
Mammalian	246.2	249.5 (6)	-
Ciona 1	256.8	257.5 (9)	248.1
Ciona D-Ala ⁶	249.9	249.8 (5)	250.7
Ciona Gly ⁶	247.7	247.6 (8)	239.4

Table III

Table showing the average results of co-ordinate analysis from extended dynamics calculations. Listed are average N-C distances ($C\alpha-C\alpha$), the distance between Trp³ and Leu⁷, and the collision cross-sections of the confirmation from co-ordinates averaged over the 1ns of dynamics performed at 300K. The figure given is averaged from those obtained from two molecular dynamic runs. The Standard Deviation from this value is given in parentheses.

		Ciona 1		DAla ⁶ Ciona 1		Gly ⁶ Ciona 1		Mammalian GnRH	
		[M]	[M+H] ⁺	[M]	[M+H] ⁺	[M]	[M+H] ⁺	[M+H] ⁺	[M+2H] ²⁺
N-C distance (Å)	<i>in vacuo</i>	-	5.8 (0.48)	-	5.5 (0.36)	-	5.4 (0.3)	7.3 (0.68)	6.2 (0.30)
	Born-Solvent	13.9 (3.7)	15.7 (3.2)	11.2 (3.27)	15.1 (3.0)	7.6(2.4)	9.3 (3.3)	9.6 (3.3)	9.2 (2.6)
Trp-Leu distance (Å)	<i>in vacuo</i>	-	7.6 (0.88)	-	7.1 (0.65)	-	6.2 (0.43)	4.5 (0.82)	5.8 (0.45)
	Born-Solvent	7.3 (1.36)	7.5 (0.84)	7.5 (1.91)	7.8 (1.98)	7.5 (2.33)	7.4 (1.08)	7.0 (1.04)	6.2 (0.65)
Collision Cross-section (Å ²)	<i>in vacuo</i>	-	259.3 (5.4)	-	250.5 (6.3)	-	247.0 (3.8)	252.2 (5.3)	258.5 (5.3)
	Born-Solvent	275.9 (12.2)	290.9 (9.7)	273.3 (11.8)	277.4 (8.6)	266.5 (5.4)	278.3 (9.2)	282.7 (11.4)	289.4 (11.4)

Figure 1

	1	2	3	4	5	6	7	8	9	10			
Jawed Vertebrates	Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	NH ₂	
	Guinea Pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly	NH ₂	
	Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly	NH ₂	
	Rana d.	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly	NH ₂	
	Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly	NH ₂	
	Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly	NH ₂	
	Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly	NH ₂	
	Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly	NH ₂	
	Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly	NH ₂	
	Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly	NH ₂	
	Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	NH ₂	
	Jawless Fish	Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly	NH ₂
		Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly	NH ₂
Protochordates/Tunicates	Chelyosoma I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly	NH ₂	
	Chelyosoma II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly	NH ₂	
	Ciona I	pGlu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	Gly	NH ₂	
	Ciona II	pGlu	His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	Gly	NH ₂	
	Ciona III	pGlu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	Gly	NH ₂	
	Ciona IV	pGlu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	Gly	NH ₂	
	Ciona V	pGlu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	Gly	NH ₂	
	Ciona VI	pGlu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	Gly	NH ₂	
	Ciona VII	pGlu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	Gly	NH ₂	

Figure 2

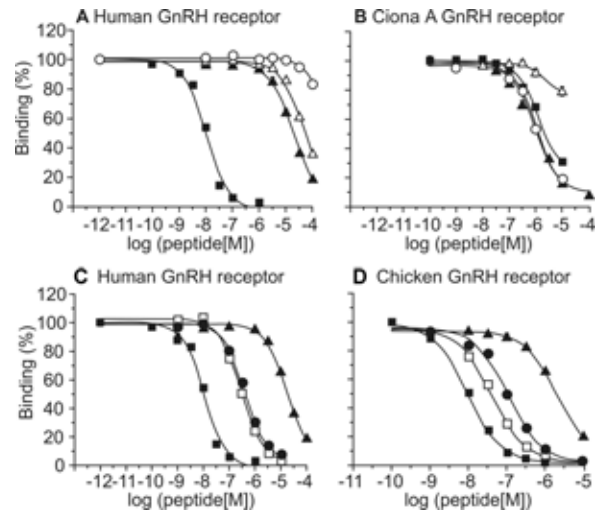


Figure 3

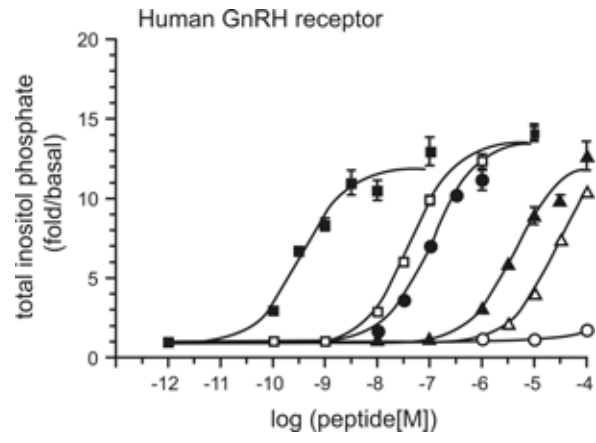


Figure 4

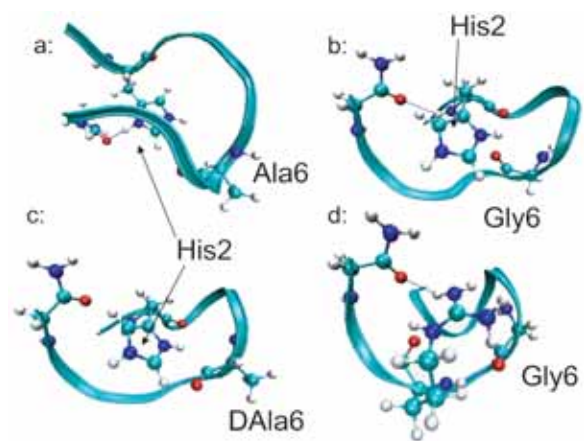


Figure 5

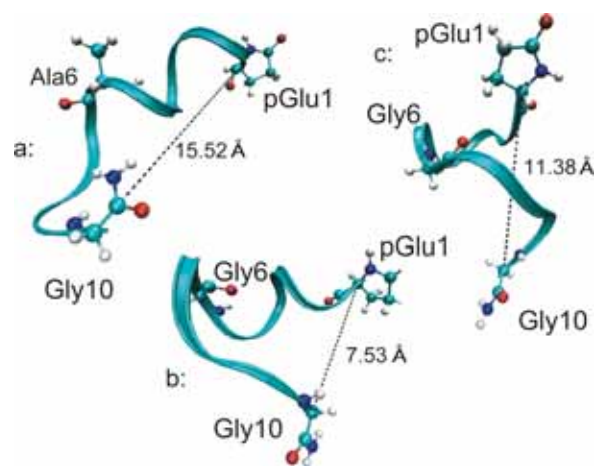


Figure 6

