0026-895X/94/020165-06$03.00/0

MOLECULAR PHARMACOLOGY, 45:165-170

ACCELERATED COMMUNICATION

A Reciprocal Mutation Supports Helix 2 and Helix 7 Proximity in the Gonadotropin-Releasing Hormone Receptor

WEI ZHOU, COLLEEN FLANAGAN, JUAN A. BALLESTEROS, KAREL KONVICKA, JAMES S. DAVIDSON, HAREL WEINSTEIN, ROBERT P. MILLAR, and STUART C. SEALFON

Dr. Arthur M. Fishberg Research Center in Neurobiology (W.Z., S.C.S.), Department of Physiology and Biophysics (J.A.B., K.K., H.W.), and Department of Neurology (S.C.S.), Mount Sinai School of Medicine, New York, New York 10029, and Medical Research Council Regulatory Peptides Research Unit, University of Cape Town Medical School, Observatory 7925, South Africa (C.F., J.S.D., R.P.M.)

Received September 23, 1993; Accepted November 10, 1993

SUMMARY

Activation of the pituitary gonadotropin-releasing hormone receptor, a member of the seven-transmembrane G protein-coupled receptor (GPCR) family, triggers a cascade of events leading to gonadotropin release and stimulation of the reproductive system. An unusual feature of this receptor, observed in mice, rats, and humans, is the presence of Asn^{87} in the second putative transmembrane helix at the location of a highly conserved asparate in the GPCR family and of Asp^{318} in the putative seventh transmembrane helix where nearly all other GPCRs have asparagine. The possibility that these residues interact was suggested by this reciprocal pattern and by a three-dimensional model of the gonadotropin-releasing hormone receptor and was investigated by site-directed mutagenesis. Replacing Asn^{87} in the second transmembrane domain by aspartate eliminated detectable ligand binding. A second mutation, generating the double-mutant receptor $Asp^{87}Asn^{318}$, recreated the arrangement found in other GPCRs and re-established high affinity agonist and antagonist binding. The restoration of binding by a reciprocal mutation indicates that these two specific residues in helices 2 and 7 are adjacent in space and provides an empirical basis to refine the model of the transmembrane helix bundle of the receptor.

The GPCRs comprise a large family of receptor proteins that mediate signaling by coupling to G proteins (1). All of the cloned GPCRs have seven hydrophobic domains that are considered to represent the transmembrane domains (TMH 1–7) and contain consensus amino acid motifs at corresponding positions (2). Residues highly conserved among all GPCRs, including receptors with widely divergent ligand structures and different coupling specificities to G proteins, are likely to be essential structural determinants of receptor function. Two such residues are an aspartate in TMH 2 and an asparagine in TMH 7, which are 98% and 95% conserved, respectively (2). However, these residues are not conserved in the mammalian GnRHR (3–5) and, in fact, appear to be interchanged (Fig. 1).

The inversion of the conserved residues suggests that this receptor may represent a natural reciprocal mutation, relative to other GPCRs, and that an interaction between these two residues contributes to the structural organization and functional integrity of the protein. To test this hypothesis, three mutant receptors, i.e., an Asp^{87} mutant, an Asn^{318} mutant, and an $Asp^{87}Asn^{318}$ reciprocal mutant, were produced and their properties were compared with those of the wild-type GnRHR expressed in COS-1 cells.

Materials and Methods

Generation and expression of mutant constructs. The mouse GnRHR cDNA (3) was subcloned into pSelect (Promega, Madison, WI) and mutations were introduced by oligonucleotide-mediated mutagenesis. For expression in COS-1 cells, the inserts from sequence-identified mutants were excised and subcloned into pcDNAI/Amp (Invitrogen, San Diego, CA). The mutations were confirmed by sequencing both strands of the inserts in the final expression vector constructs. The wild-type and mutant receptors were transiently expressed in COS-1 cells by transfection of constructs using DEAE-dextran, as described (5).

Receptor binding. Eight to 15 μg of DNA were transfected in 10-cm dishes containing 3×10^6 COS-1 cells (5, 6). Cell membranes were prepared by homogenization (Dounce) in binding buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.1% bovine serum albumin) and were

This work was supported by National Institutes of Health Grant DK44943 (S.C.S., H.W., R.P.M.), National Institute on Drug Abuse Grant K05 DA0006-13 (H.W.), Fulbright/Ministry of Education and Science-Spain fellowship (J.A.B.), and Medical Research Council, Cancer Association, South Africa, and University of Cape Town research grants (R.P.M.).

ABBREVIATIONS: GPCR, G protein-coupled receptor; GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; TMH, transmembrane helix; GnRH-A, gonadotropin-releasing hormone agonist (D-Ala^6-*N*-Me-Leu^7-Pro^9-*N*-ethylamide-gonadotropin-releasing hormone); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

a

Receptor name	HELIX 2	HELIX 7
	80 87 90 100	310 318 325
	...|.....|..|.....|....	.....|.....|.....|..
GnRH receptor (Mus musculus)	KVLLKHLTLANLLETLIVMPLDGMWNIT	NHFFFLFAFLNPCFDPLIYGYFSL
Cholecystokinin receptor (Homo sapiens)	NAFLLSLAVSDLLLAVACMPFTLLPNLM	ISFIHLLSYASACVNPLVYCFMHR
GRP/bombesin receptor (Mus musculus)	NLFISSLALGDLLLLVTCAPVDASKYLA	SICAHLLAFTNSCVNPFALYLLSK
Neuromedin B receptor (Homo sapiens)	NIFISNLAAGDLLLLLTCVPVDASRYFF	TLVARVLSFGNSCVNPFALYLLSE
Neurotensin receptor (Homo sapiens)	HYHLGSLALSDLLTLLLAMPVELYNFIW	YMVTNALFYVSSTINPILYNLVSA
5HT1C receptor (Homo sapiens)	NYFLMSLAIADMLVGLLVMPLSLLAILY	LNVFVWIGYVCSGINPLVYTLFNK
beta-2 adrenergic receptor (Homo sapiens)	NYFITSLACADLVMGLAVVPFGAAHILM	YILLNWIGYVNSGFNPLIYCRSPD

b

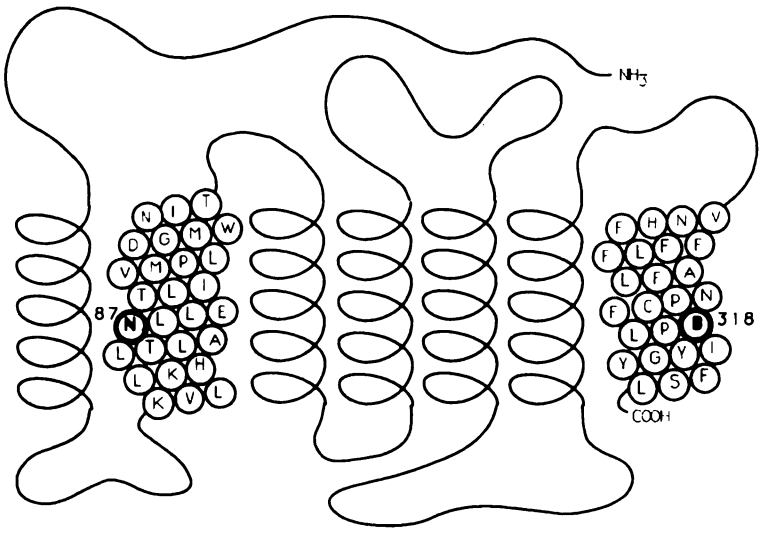

Fig. 1. Alignment of GnRHR sequences with other representative GPCR sequences, illustrating the exchange of the conserved TMH 2 aspartate and TMH 7 asparagine. a, TMH 2 and TMH 7 sequence alignments. Asn^{87} and Asp^{318} in the GnRHR are in *bold type*. *GRP*, gastrin-releasing peptide; *5HT*, 5-hydroxytryptamine. b, Helical net schematic diagram of TMH 2 and TMH 7 of the GnRHR, showing the putative locations of the residues studied by mutagenesis.

centrifuged at 15,000 × *g* for 30 min at 4°. The membrane pellet was resuspended in binding buffer and incubated for 60 min at 4° with ^{125}I-GnRH-A and varying concentrations of unlabeled GnRH, Gln^8-GnRH, GnRH-A, or GnRH antagonist 26 (Ac-D-4-Cl-$Phe^{1,2}$-D-Trp^3-D-Lys^6-D-Ala^{10}-NH_2). Binding (B_o) in mutant receptors is expressed as the mean ± standard error of the percentage of wild-type binding of two to four experiments, each done in triplicate.

Inositol phosphate production. Inositol phosphate production was assayed as described (7). A total of 2.5 μg of DNA were used for 1.8×10^6 cells in 12-well plates. Transfected cells were labeled overnight with [^{3}H]inositol and stimulated with varying concentrations of GnRH in the presence of LiCl. The reaction was terminated by the addition of perchloric acid and phytic acid. After neutralization with KOH, the inositol phosphates were separated on a Dowex ion exchange column and counted. The mean maximum inositol phosphate production obtained from dose-response curves for mutant receptors was expressed as a percentage of the inositol phosphate production by the wild-type receptor in the same experiment. Maximal stimulation of the wild-type receptor induced phosphoinositol turnover of 7.0 ± 1.0 times basal levels (three experiments).

Molecular modeling. The model of the TMH bundle of the GnRHR was constructed according to the criteria and procedures described elsewhere (8, 9), using structural inferences derived from the analysis of sequence conservation patterns (10, 11), the physico-chemical properties of conserved and partially conserved residues (12, 13), and specific protein motifs such as Pro-kinks (14–16). The predicted helix boundaries take into account the role of arginine and lysine residues at the membrane-cytoplasm interface, where these residues belong to the TMH and act as anchors to the membrane through ionic pairing with phospholipid head-groups (15). Sequence alignments were generated using the Oxford Molecular Serratus software package, and the model was refined by energy minimization using the Quanta/CHARMm molecular modeling package (Molecular Simulation, Inc.)

Results and Discussion

With expression of the Asp^{87} mutant, binding was not detectable with either labeled agonist or antagonist. Mutation of the aspartate in TMH 7 to Asn^{318} in conjunction with the TMH 2 Asp^{87} mutation ($Asp^{87}Asn^{318}$) restored high affinity binding of both agonist and antagonist to values that were similar to those obtained with the wild-type receptor (Fig. 2; Table 1).

The absence of detectable binding after expression of a receptor with a single mutation might be due to elimination of a direct ligand contact site. A direct role in ligand binding for Asn^{87}, however, is unlikely for the following reasons. (i) The residue at this locus is highly conserved among GPCRs with structurally unrelated ligands. (ii) All ligand contact sites that have been suggested from experiments are located nearer to the extracellular surface. Ligands covalently bound to the β-adrenergic receptor, for example, identify a TMH 2 attachment site located 14 residues above the corresponding GnRHR locus studied (17). (iii) As reported here, agonist and antagonist affinities were nearly identical for the wild-type receptor and the $Asp^{87}Asn^{318}$ mutant. The agonists and antagonists evaluated must have multiple contact points on the receptor, which should be asymmetrically distributed. Thus, if Asn^{87} were a direct ligand contact site, then the double mutant, introducing an asparagine in a different location, would not be expected to reconstitute a binding site with properties indistinguishable from those of the wild-type receptor.

Thus, the loss of binding of the Asp^{87} mutant must be due to a structural perturbation of the receptor that either distorts the binding site or, as determined by immunofluorescence micros-

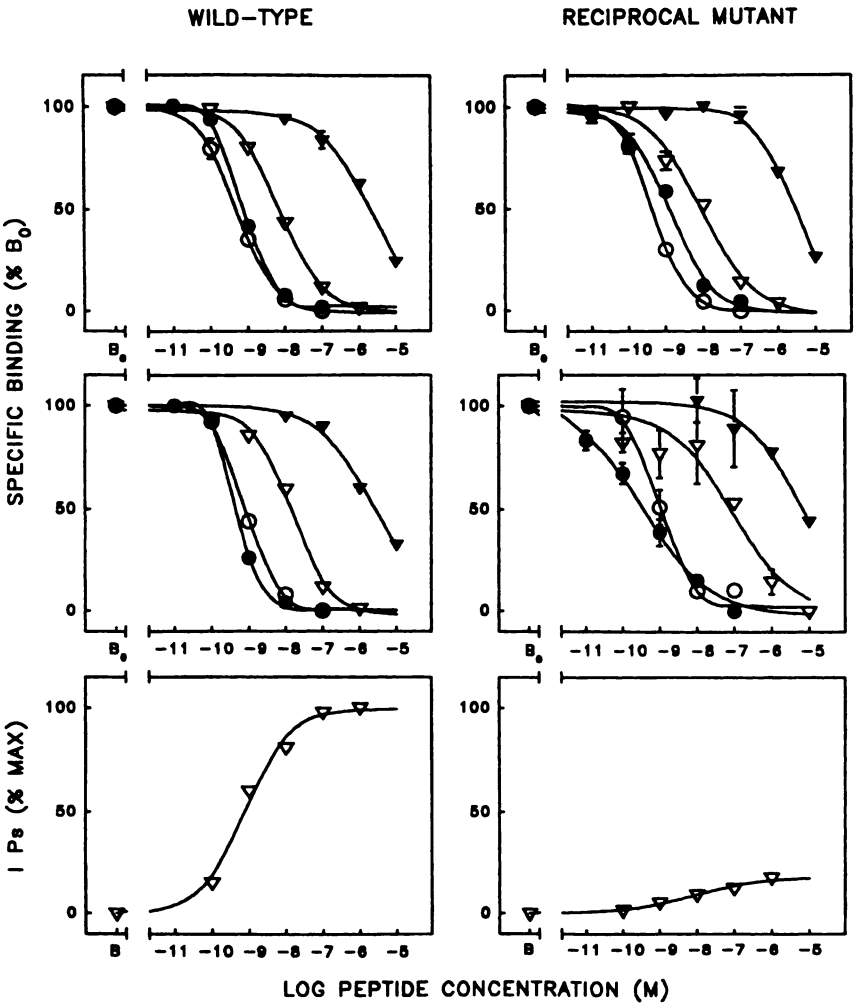

Fig. 2. Receptor binding and ligand stimulation of inositol phosphate production in COS-1 cells transfected with wild-type (*left*) and $Asp^{87}Asn^{318}$ reciprocal-mutant (*right*) receptor constructs. *Upper*, competition binding of GnRH peptides with ^{125}I-GnRH-A. ○, GnRH-A; ●, antagonist 26; ▽, GnRH; ▼, Gln^8-GnRH. *Middle*, competition binding of GnRH peptides with ^{125}I-GnRH antagonist 26. *Lower*, stimulation by GnRH of total inositol phosphate (*IPs*) production.

TABLE 1

Binding of wild-type and mutant receptors expressed in COS-1 cells

Wild-type and mutant receptors were generated and expressed as described in Materials and Methods. The ^{125}I-labeled GnRH agonist used was GnRH-A; competition data were obtained with unlabeled GnRH, Gln^8-GnRH, GnRH-A, and GnRH antagonist 26.

Construct	B_0	IC_{50}			
		GnRH	GnRH-A	Antagonist 26	Gln^8-GnRH
	%		nM		
Wild-type	100	15.9 ± 5.6	1.3 ± 0.7	2.7 ± 1.5	2605 ± 222
Asp^{87}	0.9 ± 2.1				
$Asp^{87}Asn^{318}$	39.7 ± 8.2	21.7 ± 16.7	1.3 ± 0.4	2.3 ± 1.4	4120 ± 2030
Asn^{318}	69.7 ± 7.4	10.1 ± 1.4	0.6 ± 0.2	0.9 ± 0.4	1950 ± 640

copy for an α/β_2-adrenergic receptor mutant, disrupts proper membrane insertion of the mutant receptor (18). The present data do not allow discrimination between intracellular retention of the mutant receptor and membrane expression of a receptor that does not form a functional binding pocket. However, either explanation for the loss of binding supports the conclusion that the asparagine-aspartate single mutation in TMH 2 interferes with proper packing of the membrane helices and alters the essential overall structure of the receptor.

The restoration of binding to the Asp^{87} mutant with the introduction of the reciprocal Asn^{318} mutation indicates that these residues have a complementary role in maintaining the structure of the receptor. A correlation between restoration of functional attributes by a reciprocal double mutation and spatial proximity of the targeted residues has been demonstrated with a zinc finger protein using two-dimensional NMR spectroscopy (19). Structural implications of revertant double mutants have been evaluated in terms of free energy considerations (20, 21). Regaining binding with the double mutant indicates that the changes in the free energy of binding ($RT\ln K_d$) in each single mutant are not simply additive in the double mutant but the effect of one mutation is dependent on the residue at the other locus. Such nonadditivity is expected if a single mutation causes a structural perturbation in the microenvironment of the other residue (20). It is therefore likely that Asn^{87} and Asp^{318} share a common microenvironment, a condition that could be fulfilled by direct hydrogen bonding, as illustrated in Fig. 3A, but may involve more complex networks of interacting side chains in the two helices. Furthermore, whereas the present results indicate that Asn^{87} and Asp^{318} are adjacent in space and

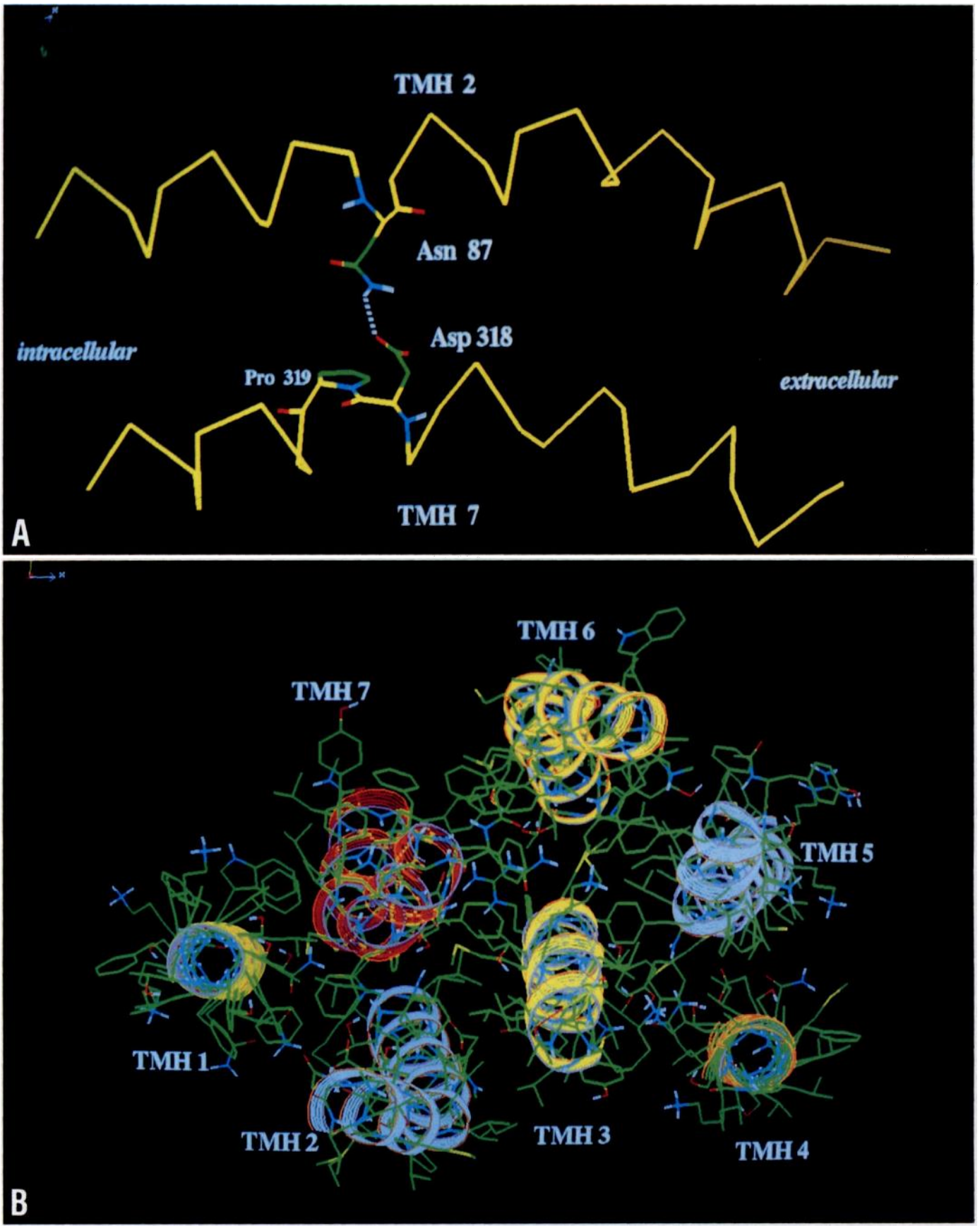

Fig. 3. Molecular modeling of the GnRHR. A, View parallel to the membrane of a partial GnRHR model, showing spatial proximity of Asn^{87} in TMH 2 and Asp^{318} in TMH 7. Note that direct hydrogen bonding has been selected for illustrative purposes only. Other hydrogen-bonding patterns and interactions are also consistent with the present experimental data. B, Three-dimensional model of the transmembrane portion of the GnRHR, viewed from the extracellular side.

therefore interact, they do not prove that the residues are directly hydrogen bonded.

Interhelical interactions have been studied in adrenergic receptors by substitution of a different TMH 7 locus that corresponds to Phe^{306} in the GnRHR. A mutation of Asn^{312} in the β_2-adrenergic receptor to phenylalanine, which is found at this position in the α_2-adrenergic receptor, eliminates function and disrupts proper membrane localization (18). The effect of this mutation, which presumably interferes with proper helix/helix packing, was corrected by exchanging both TMH 1 and TMH 2 with the homologous domains of the α_2-adrenergic receptor (22). These reports, together with the present data, are consistent with the proximity of TMH 2 and TMH 7 in the GPCRs.

The finding that the side chains of Asn^{87} and Asp^{318} are in close proximity provides an empirical structural guide for assembling the model of the receptor. Fig. 3B presents a model of the helix bundle in the transmembrane portion of the GnRHR constructed from an extensive set of considerations and criteria (8, 9). The GnRHR template resembles the projection density map of rhodopsin (13, 23), and the proposed interactions between TMH 2 and 7 are consistent with side chain interactions predicted from the model of the TMH bundle of the 5-hydroxytryptamine type 2 receptor reported recently (9).

The single mutation to Asn^{318} had no marked effect on binding (Table 1). The result that binding in the GnRHR is disrupted by a single Asp^{87} mutation but not by a single Asn^{318} mutation is consistent with the proposed structural model (Fig. 3). Aspartate is assumed to be ionized and in a hydrogen-bond interaction could only be an acceptor at the ϵ position of its side chain. Asparagine can act as both a hydrogen-bond acceptor (C—O) and a donor (NH_2) at the ϵ position. Thus, both the double-mutant receptor and the Asn^{318} receptor would allow favorable interactions such as hydrogen bonding to occur. In the Asp^{87} mutant, however, the side chains would electrostatically repulse and impair binding through disruption of receptor structure.

Although the data indicate that the binding pocket of the wild-type and reciprocal-mutant receptors are similar, the double mutant (Fig. 2) and the TMH 7 Asn^{318} mutant (data not shown) are poorly coupled to phosphoinositol turnover, compared with the wild-type receptor. The differences in coupling suggest that receptor activation requires other loci on the receptor to interact with one or both of these residues, an arrangement not replicated in the mutant receptors. The coordinated mutation of other highly conserved GPCR loci may reveal such interactions and help to elucidate the structural changes that accompany receptor activation.

The TMH 2 aspartate conserved in virtually all other GPCRs (Asn^{87} in the GnRHR) has been extensively studied by mutagenesis of neurotransmitter receptors. Replacement of this residue in different receptors has been found to have a variety of functional effects, including reduced agonist affinity (24–30), loss of modulation of binding by pH (24), by sodium (24, 31), or by GTP analogues (27, 29, 32), and diminished or absent coupling (25, 27, 32, 33). Although the functional changes reported in different receptors are diverse, they are all consistent with an alteration in the native or allosterically modulated structure of these receptors associated with the loss of the acidic aspartate side chain at this position. Our results, which indicate the proximity of this locus to a specific TMH 7 residue in the GnRHR, suggest the latter site as a novel locus to be probed in attempts to clarify the structural basis of the complex effects reported for mutation of the conserved aspartate in TMH 2 of GPCRs. To our knowledge only one study has reported the effects of substitution of the same TMH 7 asparagine. Replacing Asn^{396} in the serotonin 5-hydroxytryptamine type 1A receptor with alanine, phenylalanine, or valine eliminated agonist binding, whereas binding was retained with Gln^{396} (30). These results are consistent with the present data and the preliminary receptor model in Fig. 3B.

In the absence of detailed structural data on any GPCR, there is a paucity of experimental information from which to infer intramolecular contacts. Our results provide data supporting the proximity of specific residues in different helices. Additional potential sites of interaction between side chains in different helices can be similarly identified from the sequence and the three-dimensional model and tested to elicit experimental validation of an increasingly reliable molecular model of the GnRHR and other GPCRs.

Acknowledgments

We thank Drs. James L. Roberts, Roman Osman, and Terry Davies for critical reading of the manuscript.

References

1. Kobilka, B. Adrenergic receptors as models for G protein-coupled receptors. *Annu. Rev. Neurosci.* **15**:87–114 (1992).
2. Probst, W. C., L. A. Snyder, D. I. Schuster, J. Brosius, and S. C. Sealfon. Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol.* **11**:1–20 (1992).
3. Tsutsumi, M., W. Zhou, R. P. Millar, P. L. Mellon, J. L. Roberts, C. A. Flanagan, K. Dong, B. Gillo, and S. C. Sealfon. Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. *Mol. Endocrinol.* **6**:1163–1169 (1992).
4. Kaiser, U. B., D. Zhao, G. R. Cardona, and W. W. Chin. Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. *Biochem. Biophys. Res. Commun.* **189**:1645–1652 (1992).
5. Chi, L., W. Zhou, A. Prikhozhan, C. Flanagan, J. S. Davidson, M. Golembo, N. Illing, R. P. Millar, and S. C. Sealfon. Cloning and characterization of the human GnRH receptor. *Mol. Cell. Endocrinol.* **91**:R1–R6 (1993).
6. Millar, R. P., C. A. Flanagan, R. C. DeL. Milton, and J. A. King. Chimeric analogues of vertebrate gonadotropin-releasing hormones comprising substitutions of the variant amino acids in positions 5, 7, and 8. *J. Biol. Chem.* **264**:21007–21013 (1989).
7. Davidson, J. S., I. K. Wakefield, U. Sohnius, P. A. vanderMerwe, and R. P. Millar. A novel extracellular nucleotide receptor coupled to phosphoinositidase C in pituitary cells. *Endocrinology* **126**:80–87 (1990).
8. Ballesteros, J. A., and H. Weinstein. Construction of a model for the transmembrane domain of the serotonin $5HT_{1C}$ receptor. *Protein Sci.* **2**:1144 (1993).
9. Zhang, D., and H. Weinstein. Signal transduction by a 5-HT_2 receptor: a mechanistic hypothesis from molecular dynamics simulations of the 3-D model of the receptor complexed to ligands. *J. Med. Chem.* **36**:934–938 (1993).
10. Lesk, A. M., and D. R. Boswell. Homology modelling: inferences from tables of aligned sequences. *Curr. Opin. Structural Biol.* **2**:242–247 (1992).
11. Donnelly, D., M. S. Johnson, T. L. Blundell, and J. Saunders. An analysis of the periodicity of conserved residues in sequence alignments of G-protein coupled receptors. *FEBS Lett.* **251**:109–116 (1989).
12. Eisenberg, D., R. M. Weiss, and T. C. Terwilliger. The hydrophobic moment detects periodicity in protein hydrophobicity. *Proc. Natl. Acad. Sci. USA* **81**:140–144 (1984).
13. Baldwin, J. M. The probable arrangement of the helices in G protein-coupled receptors. *EMBO J.* **12**:1693–1703 (1993).
14. Williams, K. A., and C. M. Deber. Proline residues in transmembrane helices: structural or dynamic role? *Biochemistry* **30**:8919–8923 (1991).
15. Ballesteros, J. A., and H. Weinstein. Analysis and refinement of criteria for predicting the structure and relative orientations of transmembranal helical domains. *Biophys. J.* **62**:107–109 (1992).
16. Sankararamakrishnan, R., and S. Vishveshwara. Geometry of proline-containing *alpha*-helices in proteins. *Int. J. Peptide Protein Res.* **39**:356–363 (1992).
17. Dohlman, H. G., M. G. Caron, C. D. Strader, N. Amlaiky, and R. J. Lefkowitz. Identification and sequence of a binding site peptide of the *beta* 2-adrenergic receptor. *Biochemistry* **27**:1813–1817 (1988).
18. Suryanarayana, S., D. A. Daunt, M. Von Zastrow, and B. K. Kobilka. A point

mutation in the seventh hydrophobic domain of the α_2-adrenergic receptor increases its affinity for a family of β receptor antagonists. *J. Biol. Chem.* **266**:15488–15492 (1991).
19. Weiss, M. A., and H. T. Keutmann. Alternating zinc finger motifs in the male-associated protein ZFY: defining architectural rules by mutagenesis and design of an "aromatic swap" second-site revertant. *Biochemistry* **29**:9808–9813 (1990).
20. Carter, P. J., G. Winter, A. J. Wilkinson, and A. R. Fersht. The use of double mutants to detect structural changes in the active site of the tyrosyl-tRNA synthetase (*Bacillus stearothermophilus*). *Cell* **38**:835–840 (1984).
21. Ward, W. H., D. Timms, and A. R. Fersht. Protein engineering and the study of structure-function relationships in receptors. *Trends Pharmacol. Sci.* **11**:280–284 (1990).
22. Suryanarayana, S., M. von Zastrow, and B. K. Kobilka. Identification of intramolecular interactions in adrenergic receptors. *J. Biol. Chem.* **267**:21991–21994 (1992).
23. Schertler, G. F., C. Villa, and R. Henderson. Projection structure of rhodopsin. *Nature (Lond.)* **362**:770–772 (1993).
24. Neve, K. A., B. A. Cox, R. A. Henningsen, A. Spanoyannis, and R. L. Neve. Pivotal role for aspartate-80 in the regulation of dopamine D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol. Pharmacol.* **39**:733–739 (1991).
25. Wang, C. D., M. A. Buck, and C. M. Fraser. Site-directed mutagenesis of α_{2A}-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol. Pharmacol.* **40**:168–179 (1991).
26. Strader, C. D., I. S. Sigal, R. B. Register, M. R. Candelore, E. Rands, and R. A. Dixon. Identification of residues required for ligand binding to the *beta*-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**:4384–4388 (1987).
27. Chung, F.-Z., C.-D. Wang, P. C. Potter, J. C. Venter, and C. M. Fraser. Site-directed mutagenesis and continous expression of human β-adrenergic receptors. *J. Biol. Chem.* **263**:4052–4055 (1988).
28. Fraser, C. M. Site-directed mutagenesis of β-adrenergic receptors: identification of conserved cysteine residues that independently affect ligand binding and receptor activation. *J. Biol. Chem.* **264**:9266–9270 (1989).
29. Wang, C.-D., T. K. Gallaher, and J. C. Shih. Site directed mutagenesis of the serotonin 5-hydroxytryptamine$_2$ receptor: identification of amino acids necessary for ligand binding and receptor activation. *Mol. Pharmacol.* **43**:931–940 (1993).
30. Chanda, P. K., M. C. W. Minchin, A. R. Davis, L. Greenberg, Y. Reilly, W. H. McGregor, R. Bhat, M. D. Lubeck, S. Mizutani, and P. P. Hung. Identification of residues important for ligand binding to the human 5-hydroxytryptamine$_{1A}$ serotonin receptor. *Mol. Pharmacol.* **43**:516–520 (1993).
31. Horstman, D. A., S. Brandon, A. L. Wilson, C. A. Guyer, E. J. Cragoe, and L. E. Limbird. An aspartate conserved among G-protein receptors confers allosteric regulation of α_2-adrenergic receptors by sodium. *J. Biol. Chem.* **265**:21590–21595 (1992).
32. Surprenant, A., D. A. Horstman, H. Akbarali, and L. E. Limbird. A point mutation of the *alpha2*-adrenoceptor that blocks coupling to potassium but not calcium currents. *Science (Washington D. C.)* **257**:977–980 (1992).
33. Fraser, D. M., C.-D. Wong, D. A. Robinson, J. D. Gocayne, and J. C. Venter. Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. *Mol. Pharmacol.* **36**:840–847 (1990).

Send reprint requests to: Stuart C. Sealfon, Fishberg Research Center in Neurobiology, Mount Sinai School of Medicine, Box 1065, One Gustave L. Levy Place, New York, NY, 10029.