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The Functional Microdomain in Transmembrane Helices 2 and 7 Regulates Expression, Activation, and Coupling Pathways of the Gonadotropin-releasing Hormone Receptor*

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Structural microdomains of G protein-coupled receptors (GPCRs) consist of spatially related side chains that mediate discrete functions. The conserved helix 2/helix 7 microdomain was identified because the gonadotropin-releasing hormone (GnRH) receptor appears to have interchanged the $Asp^{2.50}$ and $Asn^{7.49}$ residues which are conserved in transmembrane helices 2 and 7 of rhodopsin-like GPCRs. We now demonstrate that different side chains of this microdomain contribute specifically to receptor expression, heterotrimeric G protein-, and small G protein-mediated signaling. An Asn residue is required in position 2.50(87) for expression of the GnRH receptor at the cell surface, most likely through an interaction with the conserved $Asn^{1.50(53)}$ residue, which we also find is required for receptor expression. Most GPCRs require an Asp side chain at either the helix 2 or helix 7 locus of the microdomain for coupling to heterotrimeric G proteins, but the GnRH receptor has transferred the requirement for an acidic residue from helix 2 to 7. However, the presence of Asp at the helix 7 locus precludes small G protein-dependent coupling to phospholipase D. These results implicate specific components of the helix 2/helix 7 microdomain in receptor expression and in determining the ability of the receptor to adopt distinct activated conformations that are optimal for interaction with heterotrimeric and small G proteins.

The gonadotropin-releasing hormone (GnRH)[1] receptor belongs to the rhodopsin-like family of G protein-coupled receptors (GPCR) (1). This family includes the light-sensitive opsins, protease-activated receptors, and receptors for neurotransmitters, peptides, and glycoproteins. High resolution structural data have not yet been obtained for any GPCR. However, projection maps of rhodopsin, amino acid sequence alignment, and computational modeling indicate that GPCRs have 7 membrane-spanning α-helices (2–6). There is a high degree of homology within the transmembrane helices and certain amino acids are highly conserved throughout the family (2, 3, 7). This diverse family shares the common function of propagating a signal across lipid membranes and the amino acid side chains which are conserved among the GPCRs are likely to constitute key structural motifs which subserve this universal GPCR function.

Several models of GPCRs, including the GnRH receptor (4, 8), have been constructed as aids for investigating receptor structure-function relations. Molecular models of GPCRs can be used to integrate experimental observations and generate structural hypotheses. However, the complexity of these structures and the limited number of experimentally determined constraints can lead to inconsistent behavior of the models (4, 7). To overcome these limitations, we have pursued the approach of identifying discrete structural motifs within receptor models, which might constitute functional microdomains. The microdomains are characterized in detail and subsequently incorporated into whole receptor models. In the GnRH receptor, for example, this approach has recently been used to propose that the motion of the conserved $Arg^{3.50(139)}$ side chain is restricted by interaction with the conserved $Asp^{3.49(138)}$ and the presence of a β-branched, hydrophobic residue, $Ile^{3.54(143)}$ (see "Experimental Procedures" for a description of the amino acid numbering scheme). Incorporation of this microdomain into the whole receptor model suggests that receptor activation is accompanied by repositioning of the $Arg^{3.50(139)}$ side chain, allowing it to interact with the $Asp^{7.49(318)}$ side chain in transmembrane helix 7 (H7) in the activated receptor conformation (4).

A related GPCR structural motif consists of this H7 side

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[1] The abbreviations used are: GnRH, gonadotropin-releasing hormone (pGlu-His-Trp-Ser-Tyr-Glu-Leu-Arg-Pro-Gly-NH_2); ARF, ADP-ribosylation factor; BFA, brefeldin A; B_{max}, maximum binding; CCK_B, cholecystokinin B; EC_{50}, agonist concentration that produces half-maximal stimulation; E_{max}, maximum response; GPCR, G protein-coupled receptor; GnRH-A, [D-Ala[6],Pro[9]-NHEt]GnRH; IC_{50}, ligand concentration which inhibits binding of ^{125}I-GnRH-A by 50%; H, transmembrane helix; IP, inositol phosphate; PLC, phospholipase C; PLD, phospholipase D; PtdBut, phosphatidyl butanol; TRH, thyrotropin releasing hormone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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chain (usually $Asn^{7.49}$) and the conserved 2.50 residue (usually Asp) in H2. The H2/H7 microdomain was originally identified from the apparent interchange of these side chains in the GnRH receptor and its functional importance was supported by reciprocal mutagenesis studies (8). Reciprocal mutation experiments in the serotonin 5-HT_{2A} (9), thyrotropin releasing hormone (TRH) (10), μ opioid (11), and NK_2 tachykinin receptors (12) have all shown that the disruption of signal transduction observed with mutation of the $Asp^{2.50}$ side chain in H2 is restored by a second mutation in H7 that interchanges the two conserved residues.

While the inter-related roles of these H2 and H7 side chains in receptor activation (9–12) suggest that they constitute a structural and functional microdomain, this conclusion has been considered controversial (13, 14). The initial study of this microdomain in the GnRH receptor reported that the presence of an Asp residue in both loci eliminated detectable binding. This result raised the possibility that charge repulsion was responsible for the observed phenotype. However, the presence of Asp at both positions in wild-type non-mammalian GnRH receptors (15, 16) and in several other GPCRs (3) as well as in functional mutant GPCRs (9, 10, 14, 17) indicates that the side chains in this microdomain, that are compatible with function, differ among GPCRs.

For a specific receptor, the side chains of the H2/H7 microdomain may contribute to receptor expression and receptor activation and coupling to intracellular signal transduction. The molecular events that underlie receptor activation are a key question in understanding receptor function. Studies of many receptors have implicated the H2/H7 microdomain as a key component of this process (9–11, 18). The involvement of this domain in multiple distinct receptor functions may account for the different results observed in the various receptors studied. The side chain at the 7.49 locus has also recently been implicated in specifying small G protein-dependent coupling to PLD (19). In order to elucidate the role of this microdomain in the GnRH receptor, we have investigated the functional requirements of each locus in receptor expression, coupling to heterotrimeric G protein-dependent signal transduction, and coupling to the small G protein, ADP-ribosylation factor (ARF).

EXPERIMENTAL PROCEDURES

Amino Acid Residue Numbering—To allow comparison of equivalent residues in different GPCRs, amino acids in the transmembrane segments of the GnRH receptor are numbered relative to the most conserved residue of the rhodopsin-like GPCRs, as described previously (7). Thus, Asn^{87}, which is located in the position of the most conserved residue in H2, is designated $Asn^{2.50(87)}$, while Asp^{318}, which is adjacent to the most conserved residue in H7 ($Pro^{7.50(319)}$), is designated $Asp^{7.49(318)}$.

DNA Constructs, Cell Culture, and Transfection—The mutations N2.50(87)D, N2.50(87)Q, N2.50(87)A, D7.49(318)N, D7.49(318)E, and D7.49(318)A were introduced into the mouse GnRH receptor as described previously (8) using the Altered Sites Mutagenesis System (Promega, Madison, WI), while the mutations N1.50(53)A, N1.50(53)D, N1.50(53)L, and D7.49(318)L were generated using QuikChange (Stratagene, La Jolla, CA). Two epitope tags were applied to the GnRH receptor to allow detection of the receptor by Western blotting. An amino-terminal HA-tag (YPYDVPDYA) was inserted after the initial Met residue of the wild-type GnRH receptor by polymerase chain reaction. Since the mouse GnRH receptor does not have a cytosolic carboxyl-terminal domain, a carboxyl-terminal domain derived from a putative human type II GnRH receptor (20) was appended to allow addition of a carboxyl-terminal hexahistidine tag using a combination of polymerase chain reaction and multifragment subcloning into the pcDNA3 expression vector (Invitrogen, San Diego, CA). The carboxyl-terminal seven amino acids of the carboxyl-terminal domain were substituted with six histidine residues to generate a hexahistidine tag. All DNA constructs were sequenced to confirm the presence of mutations and epitope tags.

COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and transfected as described previously, using LipofectAMINE (Life Technologies Inc., Gaithersburg, MD) (21). Cells to be used for intact cell functional assays were seeded into 12- or 24-well plates the day after transfection.

Ligand Binding Assays—[D-Ala^6,Pro^9-NHEt]GnRH (GnRH-A, Bachem, Torrance, CA), was radioiodinated using IODO-GEN (Pierce Chemical Co., Rockford, IL) following published protocols (22). Whole cell binding assays were performed as described (23). Briefly, transfected cells, in 24-well plates, were incubated for at least 2 h at 4 °C with ^{125}I-GnRH-A (60,000 cpm/well) and varying concentrations of unlabeled GnRH-A or GnRH (Bachem, Torrance, CA) in a total volume of 0.4 ml/well. The incubation was terminated by removal of the medium and bound radioactivity was collected in 1 M NaOH. Nonspecific binding was determined in the presence of 10^{-7} M unlabeled GnRH-A.

Membrane binding assays were performed as described previously (24) on some low-expressing constructs because this method makes it possible to increase receptor concentration in the assay by varying the amount of membrane added to incubation tubes. Cell membranes were resuspended in protein-free binding buffer (1 mM EDTA, 10 mM HEPES, pH 7.5) and incubated for 90 min on ice with ^{125}I-GnRH-A (200,000 cpm), 0.1% bovine serum albumin and varying concentrations of GnRH-A. The reaction was terminated by filtration through GF/C filters (Brandel Inc., Gaithersberg, MD) which were presoaked in binding buffer containing 1% bovine serum albumin, and washed twice with binding buffer.

Immunoblotting—Transfected cells (9-cm dishes) were washed with phosphate-buffered saline prior to harvesting and homogenization in lysis buffer (50 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 10 μg/ml leupeptin). The homogenate was centrifuged for 10 min at 500 × *g* and 4 °C and the resulting supernatant was centrifuged at 35,000 × *g* for 20 min at 4 °C. The membrane pellet was resuspended in lysis buffer (30 μl/3 × 10^6 transfected cells) and solubilized by gentle shaking for 15 min on ice in the presence of CHAPS at a final concentration of 15 mM. The solubilization mixture was centrifuged for 30 min at 35,000 × *g* and 4 °C to remove particulate material, and the resulting supernatant was incubated with *N*-glycosidase F (Roche Molecular Biochemicals, Indianapolis, IN, 0.2 units per 20 μl of supernatant) for 30 min at 37 °C. Samples were electrophoresed under reducing conditions on NuPAGE polyacrylamide gels (Novex, San Diego, CA) according to the manufacturer's instructions and electroblotted onto nitrocellulose membranes (Hybond C Pure, Amersham Pharmacia Biotech) in the presence of 0.04% SDS. Blots were blocked for at least 1 h in blot buffer (5% non-fat dry milk, 20 mM Tris, pH 7.5, 150 mM NaCl) before incubation overnight in Tetra-His antibody (Qiagen, Valencia, CA, 0.2 μg/ml in blot buffer). Bound antibody was detected using the ECL Western blotting kit (Amersham Pharmacia Biotech).

Phosphatidylinositol Hydrolysis—Accumulation of inositol phosphates (IP) in the presence of Li^+ was determined according to published protocols (25). Transfected cells were labeled for 16 h in Dulbecco's modified Eagle's medium containing 0.5 μCi/ml *myo*-[^{3}H]inositol (NEN Life Science Products, North Billerica, MA). After washing with serum-free medium the cells were incubated for 45 min at 37 °C with varying concentrations of GnRH in the presence of 20 mM LiCl. The incubation was terminated by removal of the medium and addition of 10 mM formic acid. [^{3}H]IP was separated from the formic acid extracts on Dowex-1 ion-exchange columns and eluted with 1 M ammonium formate and 0.1 M formic acid.

PLD Assay—Activation of PLD was determined by a transphosphatidylation assay as described previously (26). Transfected cells in 12-well plates were labeled overnight with [^{3}H]palmitate in serum-free minimum essential medium. After washing with minimal essential medium containing HEPES (25 mM, pH 7.5) and 1% fatty acid-free bovine serum albumin, cells were preincubated for 30 min at 37 °C with various concentrations of brefeldin A (BFA) in HEPES-buffered minimal essential medium with 0.5% bovine serum albumin, before addition of butan-1-ol (30 mM) and GnRH (1 μM) and incubation for a further 30 min. Reactions were terminated by removal of the medium and addition of 0.5 ml of cold methanol to each well. Phospholipids were extracted and separated on Whatman LK5D thin layer chromatography plates as described (26).

Data Analysis—K_d and B_{max} values for binding of GnRH-A were determined using the LIGAND computer program (27). Protein levels were determined by the Lowry method. IC_{50} (concentration required for 50% inhibition of ^{125}I-GnRH-A binding) values for GnRH were estimated using nonlinear curve fitting (KaleidaGraph, Synergy Software, Reading, PA). EC_{50} (agonist concentration required for half-maximal response) values for IP production were calculated using KaleidaGraph. IP data were fitted to the equation $E = E_{max}/(1 + EC_{50}/D)$, where the

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TABLE I
Summary of ligand binding, immunoblot, IP accumulation, and PtdBut accumulation assays in mutant GnRH receptors

Ligand binding parameters were determined from competition binding assays using ^{125}I-GnRH-A as tracer. Data are mean ± S.E. from three to five experiments performed in triplicate. Immunoblotting was performed on epitope-tagged receptor constructs as described under "Experimental Procedures." The carboxyl-terminal hexahistidine tag was detected with the tetra-His antibody and the relative intensity of the bands is indicated by the number of + signs. IP accumulation was determined in transfected COS-1 cells labeled with myo-[^{3}H]inositol. Data are mean ± S.E. from three to five experiments performed in triplicate. The efficiency of receptor coupling to activation of PLC was calculated from the summarized data in this table using the formula described under "Experimental Procedures" and expressed as a percentage of the wild-type receptor coupling efficiency. ARF-dependent PLD activity was determined as the accumulation of PtdBut in the presence of GnRH (10^{-6} M) with and without BFA (0.2 mM) and expressed relative to the PtdBut accumulation in the absence of GnRH and BFA.

Construct	Ligand binding			Immunoblot	IP accumulation			PtdBut, maximum BFA-inhibitable
	B_{max}	K_d, GnRH-A	IC_{50}, GnRH		IP max	EC_{50}, GnRH	Coupling efficiency	
	fmol/mg protein	*nM*	*nM*	*relative intensity*	*% wt*	*nM*	*% wt*	*-fold basal*
Wild-type	582 ± 135	1.2 ± 0.6	20 ± 15	++++	100	0.85 ± 0.46	100	−1.6 ± 1.5
N2.50(87)D	Und[a]			+−	−1.5 ± 0.7			0.4 ± 0.5
N2.50(87)Q	Und			+−	2.2 ± 0.7			−0.4 ± 0.4
N2.50(87)A	Und			+−	0.1 ± 0.5			0.1 ± 0.4
N2.50(87)D/D7.49(318)N	170 ± 46	1.3 ± 0.77	32 ± 20	+++	11.7 ± 4.5	30 ± 18	3.3	3.2 ± 0.6
D7.49(318)N	386 ± 42	0.97 ± 0.23	27 ± 18	+++	38.9 ± 11.7	45 ± 29	3.8	4.7 ± 0.6
D7.49(318)E	30 ± 3	2.3 ± 1.4	33 ± 7	++	36.6 ± 6.5	20 ± 10	76.7	0.3 ± 1.0
D7.49(318)A	610 ± 156	0.71 ± 0.22	22 ± 14	+++	12.0 ± 0.1	42 ± 18	0.7	0.1 ± 0.7
D7.49(318)L	Und			++	3.6 ± 0.2			
N1.50(53)D	Und			+−	4.3 ± 3.3			
N1.50(53)A	Und			+−	4.3 ± 4.1			
N1.50(53)L	Und			+−	8.5 ± 1.6			
N1.50(53)D/D7.49(318)N	Und				1.6[b]			

[a] Constructs in which total binding was too low to estimate B_{max} are reported as undetectable.
[b] $n = 1$.

E_{max} is the maximum IP accumulation and D is the concentration of the agonist. Transient transfection of the wild-type GnRH receptor into COS-1 cells leads to expression of an appreciable level of "spare receptors" which results in an EC_{50} for IP accumulation that is significantly lower than the K_d for GnRH binding to the receptor (21). Because of the receptor reserve in the wild-type receptor and the varied expression levels of the mutant receptors, simple comparison of maximal IP accumulation does not yield an accurate measure of how well a particular receptor is activated. To facilitate comparison of mutant receptor activation, we have utilized a previously derived expression of receptor coupling efficiency, Q, which is defined as: $Q = 0.5 \times [(K_d + EC_{50})/EC_{50}] \times (E_{max}/B_{max})$ (4). IC_{50} values for GnRH were used as an approximation of K_d.

RESULTS

H2 Mutants Are Not Expressed, but Expression Is Restored in H2/H7 Reciprocal Mutant—The effects of amino acid substitutions at each locus of the H2/H7 microdomain were studied. The substitutions introduced for Asn$^{2.50(87)}$ (Gln, Asp, and Ala) were designed to test the effects of altered size, charge, and polarity on receptor function. None of the single H2 mutant constructs studied exhibited detectable ligand binding activity, IP accumulation, or ARF-dependent accumulation of phosphatidyl butanol (PtdBut) (Table I).

To determine whether the lack of ligand binding by the H2 mutants resulted from altered receptor expression, we utilized an immunoblot assay of epitope-tagged receptor constructs. The parent epitope-tagged construct had an amino-terminal HA-tag and a carboxyl-terminal domain with a hexahistidine tag (see "Experimental Procedures"). The effects of epitope tagging on receptor function were evaluated. All H2 and H7 mutant constructs were epitope-tagged and tested in ligand binding and IP accumulation assays. The tagged wild-type and mutant receptors mediated IP accumulation with EC_{50} values which were comparable to those of corresponding untagged receptors. For all constructs with measurable ligand binding, K_d values were unchanged and B_{max} values elevated with epitope tagging. As was observed for the untagged receptors, ligand binding was not detectable in the epitope-tagged N2.50(87)D and N2.50(87)A constructs (Table II). The epitope-

TABLE II
Ligand binding of epitope-tagged GnRH receptors with mutations in H2

Membranes prepared from COS-1 cells transfected with wild-type and epitope-tagged wild-type and mutant GnRH receptors were incubated with ^{125}I-GnRH-A (200,000 cpm) and increasing concentrations of unlabeled GnRH-A. Membrane protein concentrations ranged from 48 to 62 μg/ml for wild-type receptors, while 400–900 μg/ml was used for cells transfected with H2 mutant receptors and vector to enhance the sensitivity of receptor detection. The low binding of the mutant receptors prevents reliable calculation of B_{max} values, thus maximum specific binding is reported to indicate relative expression levels.

Construct	Maximum specific binding	IC_{50}, GnRH-A
	fmol/mg protein	*nM*
Wild type	86.5 ± 10.9	0.91 ± 0.05
Tagged wild type	226.3 ± 27.3	1.06 ± 0.09
Tagged N2.50(87)D	1.78 ± 0.08	
Tagged N2.50(87)Q	9.3 ± 2.2	1.23 ± 0.03
Tagged N2.50(87)A	0.05 ± 0.25	
pcDNA3	0.38 ± 0.44	

tagged N2.50(87)Q construct exhibited low, but measurable ligand binding (Table II). This tagged construct also mediated a low level of GnRH-stimulated IP accumulation (not shown). The relative receptor expression, as measured by ligand binding, and the function of the tagged receptors closely paralleled that of the untagged receptors, thus validating the use of the tagged receptors in protein expression assays.

Western blots of the epitope-tagged wild-type receptor yielded a broad band of 55–85 kDa (not shown) which was compressed to a single band at 34 kDa after deglycosylation (Fig. 1). This pattern resembles that reported for the photoaffinity-labeled GnRH receptor (28). To increase sensitivity of detection and facilitate comparison of band intensity, all receptors were deglycosylated prior to immunoblot analysis. At a level of sensitivity which yielded an intense band for the epitope-tagged wild-type receptor, only a faint signal was visible for the H2 mutants in which Asn$^{2.50(87)}$ was substituted with Asp, Gln, or Ala (Fig. 1). Thus, the low or absent binding

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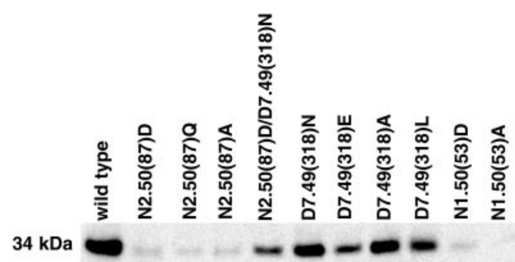

FIG. 1. **Immunoblot of GnRH receptor constructs.** COS-1 cells were transfected with epitope-tagged wild-type and mutant GnRH receptor constructs as described under "Experimental Procedures." Cell membranes were solubilized, deglycosylated, electrophoresed, and transferred to nitrocellulose membrane as described. Epitope-tagged receptors were detected with an antibody generated against tetrahistidine.

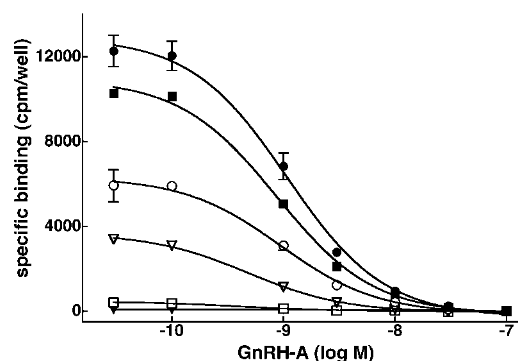

FIG. 2. **^{125}I-GnRH-A competition binding.** COS-1 cells were transfected with wild-type GnRH receptor (●) or mutant constructs D7.49(318)N (○), D7.49(318)E (□), D7.49(318)A (■), D7.49(318)L (▼), and N2.50(87)D/D7.49(318)N (▽) and incubated with ^{125}I-GnRH-A and increasing concentrations of unlabeled GnRH-A as described under "Experimental Procedures." Data are the mean ± S.E. of a representative experiment performed in triplicate.

and coupling of these constructs is associated with very low levels of receptor protein.

In contrast to the results obtained with single H2 mutants, the reciprocal mutant, N2.50(87)D/D7.49(318)N, exhibited ligand binding (Table I), as described previously (8). In addition, this receptor was also clearly visible on immunoblot analysis, yielding a band which had lower intensity than that of the wild-type receptor (Fig. 1).

Differing Expression of H7 Mutants—The functions of GnRH receptors with Asp$^{7.49(318)}$ mutated to Asn, Glu, and Ala were studied to evaluate the role of size, hydrogen bonding, and ionic interactions at this locus. All of these H7 mutants bound GnRH-A with affinities similar to that of the wild-type receptor (Fig. 2, Table I). The maximal binding of these constructs varied, with Asp (wild-type) ≥ Ala > Asn > Glu (Table I, Fig. 2). Immunoblot detection of the epitope-tagged H7 mutants correlated with the expression levels measured by ligand binding (Fig. 1). The high expression of the Ala$^{7.49(318)}$ and Asn$^{7.49(318)}$ mutants shows that the negative charge and hydrogen bonding functions of the Asp side chain are not critical for efficient receptor expression. In contrast, the D7.49(318)E mutant, which conserves the carboxylate functional group, had greatly reduced expression, both by binding (5.2% of wild-type, Table I) and by immunoblot (Fig. 1). These results indicate that the H7 interaction imposes specific steric constraints that are optimal for Asp and are only poorly matched by a negatively charged side chain of a larger size. To test whether the low expression of the Glu$^{7.49(318)}$ mutant was due to poor tolerance of the larger bulk of the Glu side chain, or to misalignment of the carboxyl group, Asp$^{7.49(318)}$ was substituted with Leu. The low expression of the D7.49(318)L mutant relative to the D7.49(318)A construct (Table I, Figs. 1 and 2) suggests that the reduced expression of the D7.49(318)E receptor results from the increase in bulk of the side chain and not from altered positioning of the carboxyl group.

H1 Mutants Are Not Expressed, and the H1/H7 Reciprocal Mutant Does Not Restore Expression—The high expression of the non-polar H7 mutant, Ala$^{7.49(318)}$, shows that the low expression of the H2 mutants is not due to loss of an interaction with the H7 side chain. It has been proposed, for other GPCRs, that the 2.50 side chain interacts with the highly conserved Asn$^{1.50}$ side chain in H1 (10, 18). To test whether the low expression of the H2 mutants might be due to disruption of an interaction with Asn$^{1.50(53)}$ in the GnRH receptor, this residue was mutated to Asp, Ala, and Leu. All H1 mutant constructs exhibited no measurable ligand binding or GnRH-stimulated accumulation of IP (Table I) and yielded only faintly detectable bands on immunoblots (Fig. 1). A reciprocal mutant, N1.50(53)D/D7.49(318)N, was constructed to test whether the locus 7.49 side chain influences the function of the Asn$^{1.50(53)}$ side chain. This reciprocal mutant showed no recovery of the ligand binding and IP accumulation which is lost in the H1, N1.50(53)D single mutant (Table I).

Phospholipase C (PLC) Activation—While all of the H7 mutants (except for Leu$^{7.49(318)}$) were capable of mediating GnRH-stimulated IP production, the EC_{50} values were increased and E_{max} values decreased in comparison with the wild-type GnRH receptor (Fig. 3*A*, Table I). The magnitude of IP stimulation observed did not correlate with levels of mutant receptor expression. For example, the D7.49(318)A construct, which expressed at wild-type levels, exhibited low maximal IP response (12% of wild-type E_{max}). In contrast, the poorly expressed D7.49(318)E mutant showed a relatively high IP signal (36.6% of wild-type E_{max}). These results reveal distinct side chain requirements for expression and for coupling to PLC.

An empirical measure of receptor coupling efficiency that estimates the functional response achieved per agonist-occupied receptor was calculated for each construct, as described previously (4) (see "Experimental Procedures"). The rank order of coupling efficiency for PLC was: wild-type, Asp > Glu > Asn > reciprocal H2D/H7N > Ala (Fig. 3*B*, Table I). It was not possible to calculate coupling efficiency for the D7.49(318)L mutant because of its lack of measurable binding and IP accumulation. The low efficiency of the Ala and Asn mutants indicates the importance of the polar and ionic functions of the native Asp side chain in PLC coupling. However, the high efficiency of the D7.49(318)E construct shows that a carboxylate side chain is required for efficient coupling to PLC.

It is notable that the PLC coupling efficiency of the reciprocal mutant (with Asp in H2, Asn in H7) was lower than for the D7.49(318)N single mutant (Table I, Fig. 3*B*). In other PLC-coupled GPCRs, mutants containing Asn residues at both the 2.50 and 7.49 loci were poorly coupled (9, 10), a result which we also see in the GnRH receptor (Table I). However, in contrast to the other GPCRs studied, where reciprocal mutation restored coupling (9, 10), the GnRH receptor appears unique in that the poor coupling persists with the interchange mutations. These commonalties and differences have implications for understanding the pattern of intramolecular signal transduction associated with GnRH receptor activation (see "Discussion"). They may also represent the special properties or importance of an Asp side chain in H7 in the GnRH receptor (18), which is present in the wild-type receptor in species from bony fish to mammals.

PLD Activation—We have recently reported that the BFA-sensitive component of PLD activation depends on receptor interaction with small G proteins in the ARF/RhoA family (19).

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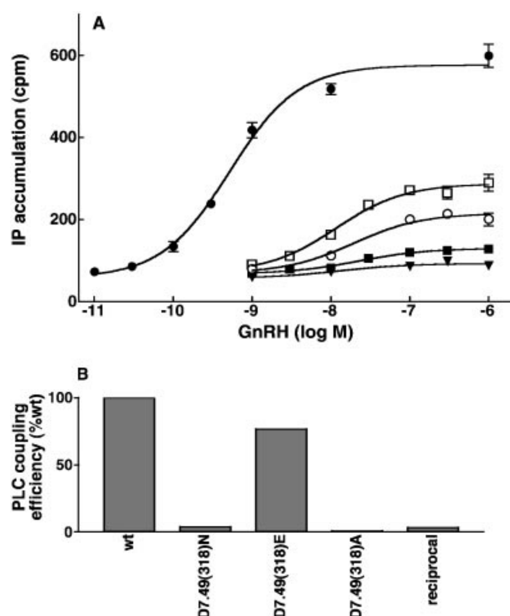

FIG. 3. **Coupling to PLC.** GnRH-stimulated accumulation of IP was measured (*top panel*) in COS-1 cells transfected with wild-type (●) and D7.49(318)N (○), D7.49(318)E (□), D7.49(318)A (■), and N2.50(87)D/D7.49(318)N (▼) mutant GnRH receptor constructs. Data are the mean ± S.E. of a representative experiment performed in triplicate. PLC coupling efficiencies (*lower panel*) were calculated from the data in Table I to facilitate comparison of receptor-mediated activation of PLC, independently of effects on receptor expression.

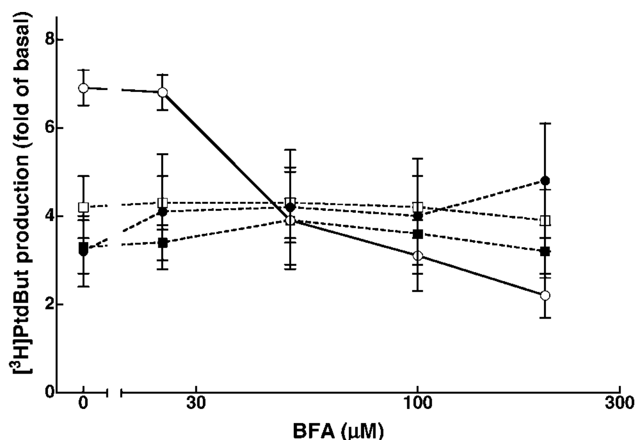

FIG. 4. **Coupling to PLD.** GnRH-stimulated accumulation of [^{3}H] PtdBut was measured in COS-1 cells transfected with wild-type (●) and D7.49(318)N (○), D7.49(318)E (□), and D7.49(318)A (■) mutant GnRH receptor constructs. Transfected cells were stimulated with GnRH (1 μM) in the presence of increasing concentrations of the ARF inhibitor, BFA. Data are the mean ± S.E. of four to ten experiments performed in duplicate.

GnRH was found to stimulate PtdBut accumulation via the wild-type receptor and all of the H7 mutants (Fig. 4, Table I). However, only the response mediated by the D7.49(318)N mutant and the N2.50(87)D/D7.49(318)N reciprocal mutant exhibited the BFA sensitivity characteristic of coupling to the small G protein, ARF (Fig. 4, Table I). Thus, in contrast to heterotrimeric G-protein coupling, coupling to ARF appears to have a stringent requirement for an Asn residue at position 7.49. This requirement may relate to stabilization of different activated receptor conformations when the side chain at this position is varied (see "Discussion").

DISCUSSION

The H2/H7 microdomain of the GnRH receptor is unusual among GPCRs in having an Asn residue at position 2.50(87). Our study of the role of each side chain of the microdomain in distinct GnRH receptor functions supports the importance of this structural microdomain and reveals the elements of the microdomain that are necessary for each function. The Asn in H2 is required for stable receptor expression and the Asp in H7 is critical both for efficient coupling to PLC and for excluding ARF-dependent coupling to PLD.

Requirement for Asn$^{2.50(87)}$ in Receptor Expression—All mutations of the Asn residue in H2 profoundly disrupted receptor expression. This resulted in a loss of signal in ligand binding assays, second messenger determination, and immunoblot analysis. The immunoblot results indicate that the loss of receptor-binding sites for the H2 mutants most likely results from a decrease in membrane receptor protein. Mutations that led to intracellular retention of receptor (29) and receptor protein instability (30) have been reported for the β-adrenergic receptor. Thus, the loss of GnRH receptors with mutations of H2 could be due either to disruption of biosynthesis and trafficking to the membrane or to instability and degradation of the expressed receptor.

The requirement for an Asn$^{2.50(87)}$ residue in H2 for expression is an unusual feature of the GnRH receptor. Furthermore, the detrimental effect of the simultaneous presence of Asp at both the 2.50 and 7.49 loci on receptor expression is not observed in most other GPCRs studied (9, 10, 12, 17, 31). Incorporation of a second Asp residue in the H2/H7 microdomain, by mutating the Asn$^{7.49}$ residue to Asp in the 5-HT$_{2A}$, TRH, cholecystokinin B (CCK$_B$), and NK$_2$ receptors, which have Asp at position 2.50, caused relatively modest decreases in receptor B_{max} levels (9, 10, 12, 14). Only the μ-opioid receptor is similar to the GnRH receptor in manifesting complete loss of binding with Asp present at both the H2 and H7 loci (11). The ability of some GPCRs to tolerate Asp side chains at both loci has led some workers to conclude that the H2 and H7 side chains are unlikely to be in close proximity (14). However, crystallographic studies reveal that Asp side chains can occur in close proximity within proteins and can form hydrogen bonds when one of the Asp side chains is protonated (32, 33). Thus, the tolerance of Asp residues at both loci in various GPCRs does not exclude proximity of the side chains of the H2/H7 microdomain. Differences in the complement of amino acids that constitute the microenvironment of this domain in different receptors most likely determine the specific side chains that are tolerated at each position. The present results and data from all other GPCRs studied are consonant with spatial proximity of the conserved H2 and H7 side chains.

In view of the profound decrease in expression observed with substitutions for Asn$^{2.50(87)}$, it is difficult to draw firm conclusions from mutagenesis studies about the role of this side chain in receptor coupling. The detection of some receptor function with the epitope-tagged N2.50(87)Q mutant suggests that preserving a polar amide side chain at this position may preserve receptor function to a greater extent than the other substitutions tested. While a role of Asn$^{2.50(87)}$ in receptor activation cannot be inferred from the present data, such a role would be consistent with previously reported computational studies which suggest that both Asn$^{2.50(87)}$ and Asp$^{7.49(318)}$ interact with the conserved Arg$^{3.50(139)}$ side chain to stabilize the active state of the receptor (4).

H7 Side Chain at the 7.49 Locus Is Not Required for Receptor Expression—We have demonstrated that restoration of GnRH receptor binding with the reciprocal mutation, N2.50(87)D/D7.49(318)N, is accompanied by restoration of receptor expression, as determined by immunoblotting. These results suggest that the strict requirement for Asn in position 2.50 can be satisfied by insertion of an amide side chain in the spatially

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adjacent 7.49 locus. Alternatively, a charged Asp side chain can substitute for the $Asn^{2.50(87)}$ side chain in the GnRH receptor when a destabilizing interaction with the H7 side chain is removed by substitution of the charged $Asp^{7.49(318)}$ residue with an uncharged Asn residue. The relatively high expression of the D7.49(318)A mutant indicates that a direct interaction between the 2.50 and 7.49 side chains is not required for receptor expression. This mutant was the best expressed of the mutant receptors in this study, but since the side chain of Ala is non-polar, it cannot form a hydrogen bond with the residue in position 2.50. It has been proposed that the $Asp^{2.50}$ side chain of the TRH receptor forms hydrogen bonds with both the $Asn^{7.49}$ side chain and the highly conserved $Asn^{1.50}$ side chain in H1 (10). The $Asn^{1.50(53)}$ residue is conserved in the GnRH receptor and, based on computational modeling (4, 8), could interact with the $Asn^{2.50(87)}$ side chain. Our results showing that an interaction with $Asp^{7.49(318)}$ is not required for expression of the GnRH receptor, suggested that an interaction between the side chains of $Asn^{2.50(87)}$ and $Asn^{1.50(53)}$ may be required for stable receptor expression. Mutation of $Asn^{1.50(53)}$ to Ala, Asp, or Leu also yielded constructs with very low expression, similar to the H2 mutants. The similar phenotypes of mutants with subtle changes in H1 or H2 is consistent with, and supports a role for a hydrophilic interaction between these side chains in stabilizing expression of the GnRH receptor. A reciprocal mutant, N1.50(53)D/D7.49(318)N, did not recover the function lost in the H1 mutants, showing that the H7 side chain does not influence the function of the H1 side chain in the same way as it does the H2 side chain. This is consistent with molecular models which show polar interactions of the 2.50 side chain with the 1.50 and 7.49 side chains, but no direct interaction between the 1.50 and 7.49 loci (7, 10, 18, 34).

The high levels of expression observed with mutations of the $Asp^{7.49(318)}$ to either Asn or Ala indicate that the functional features of the Asp side chain are not required for efficient receptor expression. In fact, the H7 mutant which preserves the acidic group, D7.49(318)E, had much lower expression than receptors with Ala or Asn substitutions at this position. These data reveal that expression is sensitive to the length of the negatively charged side chain at position 7.49. These results suggest that a larger side chain at position 7.49 may interfere with receptor assembly, either through steric interference that would disrupt helix packing due to increased bulk of the side chain, or through a disruption of protein folding by misalignment of the carboxyl group. To determine whether the detrimental effect of the $Glu^{7.49(318)}$ mutation on receptor function was due to the increased bulk of the side chain or to unfavorable positioning of the carboxyl group, $Asp^{7.49(318)}$ was mutated to Leu. The low expression of this mutant indicates that the receptor cannot accommodate a bulky side chain in this position.

Acidic Side Chain Required at Locus 7.49 for Efficient Activation of PLC—In contrast to the poor expression seen with the mutation of $Asp^{7.49(318)}$ to Glu, the PLC coupling efficiency of this mutant is comparable to that of the wild-type receptor (Table I, Fig. 3*B*). In contrast, the D7.49(318)A mutant was well expressed and yet was nearly uncoupled from PLC activation (Table I, Fig. 3). The preservation of the PLC coupling efficiency of the D7.49(318)E mutant, which conserves the negative charge of the Asp side chain, indicates that a carboxylate side chain is necessary for efficient activation of heterotrimeric G proteins. The rank order of the coupling efficiency of mutants with H7 side chain substitutions, Asp $>$ Glu $>$ Asn $>$ Ala, is consistent with the involvement of ionic and hydrogen bonds in the interactions of these side chains with the highly conserved $Arg^{3.50(139)}$ residue. These data are consistent with our proposal that an interaction between the $Arg^{3.50(139)}$ and $Asp^{7.49(318)}$ side chains stabilizes receptor activation (4).

The amino acid side chains in the H2/H7 microdomain of the GnRH receptor that are required for efficient coupling to heterotrimeric G proteins differ from those required in other GPCRs. In GPCRs which have the canonical wild-type Asp-Asn arrangement of the microdomain, the $Asp^{2.50}$ side chain is required for efficient G protein coupling (9, 10, 12, 14, 31). In addition, a polar residue is required in H7. Mutation of $Asn^{7.49}$ to Ala significantly uncoupled the β_2-adrenergic, angiotensin AT1, serotonin 5-HT_{2A}, TRH, and NK_2 tachykinin receptors (9, 10, 12, 17, 35). However, mutation of $Asn^{7.49}$ to Asp had minimal effects on the coupling of the β_2-adrenergic, serotonin 5-HT_{2A}, TRH, CCK_B, and NK_2 receptors (9, 10, 12, 14, 17). The ability of Asp, but not Ala, to substitute for the conserved $Asn^{7.49}$ residue suggests that hydrogen bonding interactions of $Asn^{7.49}$ may be required for efficient coupling of these receptors. Thus, single site mutation experiments show that activation of these GPCRs requires an acidic (Asp) residue in position 2.50 and a polar residue (Asn or Asp) in the 7.49 locus. Reciprocal mutation of this microdomain in the 5-HT_{2A}, TRH, μ-opioid, and NK_2 receptors has shown that moving the Asp side chain from H2 to H7 results in a significant recovery of the uncoupling which results from the loss of the Asp side chain at position 2.50 (9–12). Thus, for many GPCRs, the presence of an Asp in the H2/H7 microdomain is required for efficient coupling, but the Asp may be located at either position 2.50 or 7.49. In the GnRH receptor, single mutations of $Asp^{7.49(318)}$ show that this receptor also requires an acidic residue for coupling to PLC (Table I, Fig. 3). However, unlike the other GPCRs, the movement of the Asp side chain within the microdomain (from H7 to H2 in the double mutant) does not preserve efficient PLC coupling. Thus, in the GnRH receptor, the Asp residue which is required for efficient coupling appears to have been transferred from H2 to H7 in comparison with other GPCRs. Furthermore, the GnRH receptor has fixed the requirement for the acidic side chain at the H7 locus. The structural basis for these unique features of the GnRH receptor may be determined by the various non-conserved side chains that contribute to the environment of the H2/H7 microdomain, including the special properties of the H7 structure which, in the GnRH receptor, has 2 NP/DP motifs (18).

The non-mammalian GnRH receptors have Asp residues in both the H2 and H7 loci (15, 16). The presence of the two Asp residues in these receptors raises the possibility that the non-mammalian GnRH receptors represent evolutionary intermediates between the conserved Asp-Asn arrangement found in most GPCRs and the Asn-Asp arrangement found in all mammalian GnRH receptors (1). Like the mammalian GnRH receptor, the non-mammalian, catfish receptor exhibited decreased coupling when the Asp residue in H7 was mutated to Asn (15). As in the present study, lack of ligand binding activity prevented analysis of H2 mutants of the catfish GnRH receptor (15), so it is not possible to determine whether the carboxylate function of the H2 Asp side chain is required for coupling of the non-mammalian GnRH receptor. In contrast, the platelet activating factor receptor, which also has Asp residues at both loci, retained coupling to PLC when the H7 Asp residue was mutated, but was uncoupled when the H2 Asp residue was mutated (36), showing that this receptor retains its Asp residue coupling function in H2.

$Asn^{7.49(318)}$ Required for ARF-mediated Activation of PLD—Computational simulations of agonist binding to the wild-type and D2.50N mutant serotonin $5HT_{2A}$ receptors showed that agonist binding induces a conformational rearrangement of the mutant receptor which is different from the agonist-induced

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conformation of the wild-type receptor (9). This suggests that mutant GPCRs that do not have an Asp residue in the H2/H7 microdomain are able to assume an activated conformation, but that this conformation is different from the activated conformation of the wild-type receptors that have an Asp residue in this microdomain. Mutation of the $Asp^{2.50}$ side chain has variable effects on signal transduction in different receptors (9, 31). Mutating the $Asp^{2.50}$ residue of the α_2-adrenergic receptor had differential effects on different signal transduction pathways. The D2.50N mutant α_2-adrenergic receptor retained the ability to mediate inhibition of adenylyl cyclase and calcium currents, but could not achieve the conformation necessary for activation of the distinct G proteins that mediate activation of potassium channels (37).

The GnRH receptor mutants which lack an acidic residue in the H2/H7 microdomain were poorly coupled to activation of PLC. In contrast, the D7.49(318)N mutant and the N2.50(87)D/D7.49(318)N reciprocal mutant gained the capacity to mediate ARF-dependent activation of PLD (Table I, Fig. 4). Furthermore, as previously demonstrated, the reciprocal mutant (N2.50(87)D/D7.49(318)N) also shows a pattern of PtdBut accumulation characteristic of ARF-dependent signaling (19). Thus, mutant receptors which do not efficiently adopt the activated conformation necessary for activation of heterotrimeric G protein-dependent signaling were, nevertheless, able to activate the small G protein, ARF. The requirement for an Asn residue at position 7.49 for ARF-mediated coupling to PLD is shared by all GPCRs in which BFA-sensitive coupling has been studied (19). For example, a mutant of the serotonin 5-HT_{2A} receptor with Asp substituted for $Asn^{7.49}$ retained coupling to PLC, but lost BFA-sensitive coupling to PLD (19). This shows that the presence of a carboxylate side chain in the 7.49 locus prevents interaction of agonist-occupied GPCRs with the ARF·RhoA complex, regardless of the ability to activate heterotrimeric G proteins. Computational studies of the 5-HT_{2A} receptor indicate that the activated receptor conformation of the wild-type ($Asn^{7.49}$) receptor differs from that of the ARF-uncoupled N7.49D mutant in the conformation of the activated form of the receptor, but not of the inactive form.[2] These results suggest that the optimal receptor conformation which mediates heterotrimeric G protein-dependent PLC coupling differs from the conformation required for ARF-mediated PLD coupling and that the capacity to assume a conformation for coupling to ARF requires an Asn side chain at position 7.49.

We find that different side chain elements of the H2/H7 microdomain in the GnRH receptor are required for specific receptor functions. The GnRH receptor is unusual among GPCRs in requiring Asn residues both at position 2.50 and position 1.50 for receptor expression, and in its transfer of the acidic residue required for efficient PLC activation from locus 2.50 to 7.49. The presence of an Asp side chain in position 7.49 in the wild-type GnRH receptor serves to prevent ARF-dependent coupling to PLD, in common with other receptors which have an Asp residue in this position (19). These findings indicate that the H2/H7 microdomain forms a critical part of the machinery that underlies activation of the rhodopsin-like GPCRs, and that it exhibits specific structure-related properties that are revealed by comparison of the GnRH receptor with other GPCRs.

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[2] K. Konvicka and H. Weinstein, unpublished results.

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The Functional Microdomain in Transmembrane Helices 2 and 7 Regulates Expression, Activation, and Coupling Pathways of the Gonadotropin-releasing Hormone Receptor

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