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An Anionic Residue at Position 564 Is Important for Maintaining the Inactive Conformation of the Human Lutropin/Choriogonadotropin Receptor

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ABSTRACT

Gonadotropin-independent, male-limited precocious puberty is caused by a variety of mutations in the lutropin/choriogonadotropin receptor (LHR) that produce constitutive receptor activation. Two of these mutations encode replacement of conserved aspartate residues at positions 564 and 578 with glycine. We previously used site-directed mutagenesis to study the functional role of the Asp578 side chain in transmembrane helix 6, and concluded that it is its ability to serve as a properly positioned interhelical hydrogen bond acceptor, rather than its negative charge, that is important for stabilizing the inactive state of the LHR. We now report the effects of substituting seven different amino acids for the Asp564 residue located at the carboxyl terminus of the third intracellular loop. Glycine, alanine, valine, leucine, phenylalanine, and asparagine produced constitutive activation in a COS-7 cell expression system (3–5-fold increase in basal cAMP), but glutamate did not, indicating that a negative charge at position 564 may be important for maintaining the inactive LHR conformation. Characterization of double-mutant receptors showed that certain substitutions at Asp564 and Asp578 have a cumulative effect on basal receptor activity, perhaps because they mimic different aspects of the activation process normally triggered by hormone binding.

The human LHR is a GPCR that consists of a large, glycosylated amino-terminal extracellular domain connected to a bundle of seven membrane-spanning α-helical segments (Segaloff and Ascoli, 1993). The major effect of hormone binding to the extracellular domain is an increase in intracellular cAMP, but secondary coupling of the LHR to the IP pathway has also been described (Segaloff and Ascoli, 1993; Gudermann *et al.*, 1992).

The precise mechanisms by which hormone binding relays a signal to the cytoplasmic surface of the transmembrane bundle in the LHR and other GPCRs remains unknown. Inactive receptors are believed to exist in a constrained conformation that is maintained by a network of interhelical bonds. The best studied GPCR is rhodopsin, whose inactive state is characterized by a salt bridge between charged residues in TM3 and TM7. Light-induced isomerization of retinal disrupts this bridge and triggers a series of other changes, such as protonation of a highly conserved Glu residue at the cytoplasmic end of TM3 (Cohen *et al.*, 1993; Arnis *et al.*, 1994) and movement of TM6 (Farrens *et al.*, 1996) that allow rhodopsin to assume an activated conformation and couple to its G protein. The exposure of several intracellular GPCR domains, including the amino- and carboxyl-terminal ends of the i3, seems to be critical for G protein interaction (Probst *et al.*, 1992; Baldwin *et al.*, 1997).

One way to gain understanding of the mechanism by which GPCRs undergo conformational change is to study the properties of activating GPCR mutations that partially mimic the effect of agonist occupancy. Heterozygous missense mutations affecting 10 different residues in the LHR, mostly clustered in TM6 (Fig. 1), are the cause of familial and sporadic forms of gonadotropin-independent, male-limited precocious puberty, or testotoxicosis (Shenker *et al.*, 1993; Laue *et al.*, 1995; Kraaij *et al.*, 1995; Themmen and Brunner, 1996). Boys with this rare disorder exhibit autonomous Leydig cell function due to inappropriate intracellular cAMP accumulation triggered by the mutant LHR.

The most common testotoxicosis mutation encodes

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ABBREVIATIONS: LHR, lutropin/choriogonadotropin receptor; GPCR, G protein-coupled receptor; TM, transmembrane helix; i3, third intracellular loop; WT, wild-type; hCG, human chorionic gonadotropin; AR, adrenergic receptor; IP, inositol phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Asp578Gly. We previously applied site-directed mutagenesis to investigate the role that this position plays in maintaining the inactive LHR conformation (Kosugi *et al.*, 1996). Substitution of the highly conserved Asp578 with polar residues that have shorter (Ser) or longer (Glu, Tyr) side chains, or with a similarly sized hydrophobic residue (Leu), produced constitutive activation of the cAMP pathway, but substitution with an uncharged, isomorphous Asn did not (Kosugi *et al.*, 1996). These results indicate that it is the ability of Asp578 to serve as a properly positioned H-bond acceptor, rather than its negative charge, that is important for stabilizing the inactive state. Bulky aromatic side chains (Tyr, Phe) at position 578 were especially activating, an effect that may be attributed to additional disruption of interhelical packing. Only the Tyr, Phe, and Leu mutants showed constitutive activation of the IP signaling pathway.

Another activating LHR mutation encoding an Asp-to-Gly substitution has been found in codon 564 in a single sporadic case of testotoxicosis (Laue *et al.*, 1995). Asp564 is located at the carboxyl terminus of i3, near the junction with TM6 (Fig. 1), and is conserved in all glycoprotein hormone receptors. The corresponding position is occupied by an acidic Glu residue in the opsins and many other GPCRs (Probst *et al.*, 1992), suggesting that an acidic residue may play a conserved function in this group of proteins.

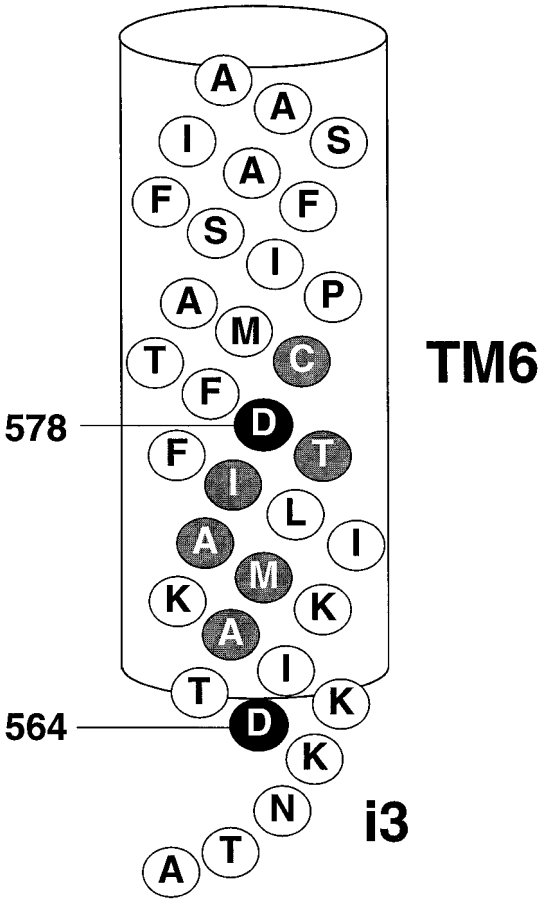

Fig. 1. Helical net representation of TM6 and the carboxyl terminus of i3 of the human LHR. Residues that are the sites of activating mutations in testotoxicosis are denoted with *black circles* (Asp564 and Asp578) or *gray circles*. According to the GPCR residue numbering system used by Baldwin *et al.* (1997), Asp578 corresponds to VI:12 and Asp564 corresponds to VI:-2.

To discern the structural basis of the activation caused by the naturally occurring Asp564Gly substitution in the LHR we used site-directed mutagenesis to substitute Gly and six other amino acids with varying chemical properties for the WT Asp. The mutant receptors were transiently expressed in COS-7 cells, and hCG binding, cAMP, and IP production were measured in intact transfected cells. We also engineered and analyzed the phenotype of three double-mutant receptors in an attempt to better understand the relationship between Asp564 and Asp578 in maintaining the inactive conformation of the LHR.

Materials and Methods

Site-directed mutagenesis of the LHR. Human LHR cDNA was inserted into the *Eco*RI site of the M13mp18 vector, and oligonucleotide-mediated site-directed mutagenesis was used to generate clones encoding the desired mutation (T7GEN kit; U. S. Biochemical, Cleveland, OH). Residue numbers were determined by counting from the methionine start site. WT and mutant clones were inserted into the *Eco*RI site of the SV-40 driven pSG5 vector (Strategene, La Jolla, CA). Mutations were confirmed by DNA sequencing of the final construct and plasmid DNA was purified by CsCl-gradient ultracentrifugation.

Transfection and assays. COS-7 cells ($\sim 10^7$ cells) were transfected by electroporation (Bio-Rad, Richmond, CA) with 25 μg of purified plasmid DNA containing a mutant or WT LHR sequence. After electroporation, each batch of transfected cells was divided into aliquots for binding, cAMP, and IP assays. Cells intended for binding assays were suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and transferred to 6-well plates ($\sim 5 \times 10^5$ cells/well). Cells for cAMP and IP assays were suspended in inositol-free medium supplemented with 10% fetal calf serum and 2.5 μCi/ml *myo*-[2-^{3}H]inositol (DuPont NEN, Boston, MA) and were transferred to 24-well plates ($\sim 10^5$ cells/well). Forty-eight hours after transfection, cells were washed with assay buffer (Hanks' balanced salt solution containing 0.5% (w/v) crystalline bovine serum albumin and 20 mM HEPES-NaOH, pH 7.4). ^{125}I-hCG binding was measured by incubating cells for 16 hr at 4° in 1 ml of assay buffer containing approximately 300,000 cpm of ^{125}I-hCG (CR-127, 14,900 IU/mg, National Hormone and Pituitary Program; labeled to about 40 μCi/μg by Hazelton Washington, Vienna, VA) and 0–10^{-7} M unlabeled hCG. cAMP and IP production were measured concurrently by incubating cells for 1 hr at 37° in 0.2 ml assay buffer containing 10 mM LiCl, 0.5 mM IBMX (3-isobutyl-1-methylxanthine) and 0–1000 ng/ml hCG. Perchloric acid was added to each well, samples were centrifuged, and total cAMP in aliquots of supernatant neutralized with KOH and HEPES was determined by ^{125}I radioimmunoassay (Eiken, Tokyo, Japan). Total IPs were measured using Dowex AG1-X8 anion exchange column chromatography (Bio-Rad). All assays were performed at least in triplicate, on at least three separate occasions with different batches of cells, and always included control cells transfected with WT LHR DNA. COS-7 cells transfected with pSG5 vector alone were not stimulated by hCG and did not exhibit specific ^{125}I -hCG binding. The program LIGAND (Munson and Rodbard, 1980) was used to estimate nonspecific binding and calculate K_d and B_{max} values for specific hCG binding. Log-transformation was used to calculate geometric means and 95% confidence limits for K_d and EC_{50} values. cAMP and IP data are expressed as fold-increase over basal in cells transfected with WT human LHR DNA (mean ± standard error of ≧ 3 experiments). The density of live cells varied <10% between wells transfected with WT LHR and those transfected with mutant constructs.

Results

Constitutive activation by Asp564Gly substitution. Fig. 2 shows results from an experiment comparing the properties of the WT and Asp564Gly (D564G) mutant LHR in COS-7 cells. The D564G substitution did not significantly affect the equilibrium dissociation constant (K_d) of the LHR for the agonist hCG, and caused only a minimal decrease in cell surface expression when the equivalent amount of LHR DNA (25 μg) was used for transfection (Fig. 2A; Table 1). COS-7 cells transfected with the WT LHR have the same basal cAMP and IP production as cells transfected with vector alone (Fig. 2, B and C) (Shenker *et al.*, 1993; Kosugi *et al.*, 1996). Cells transfected with 25 μg of D564G DNA exhibited a 3.8-fold increase in basal cAMP accumulation, an effect that has been reported previously (Laue *et al.*, 1995), but exhibited no increase in basal IP production (Fig. 2, B and C; Table 1).

Transfection of cells with lower amounts of D564G mutant DNA (10 and 3 μg) resulted in a decrease in estimated receptor density (Fig. 2A) and a proportionate decrease in hCG-stimulated cAMP (Fig. 2B) and IP production (Fig. 2C). Basal cAMP accumulation by D564G in COS-7 cells is also proportionate to receptor density, confirming that the observed activation is receptor-mediated.

Ligand binding of other Asp564 mutants. None of the six other amino acid substitutions at position 564 had a marked effect on the K_d of the LHR for the agonist hCG, or on levels of receptor expression (Table 1). The estimated surface concentrations of the mutant receptors, expressed as a percentage of WT B_{max}, ranged from 70% for the Leu mutant (D564L), to 146% for the Glu mutant (D564E).

cAMP and IP production by other Asp564 mutants. The effects of all Asp564 mutations on basal and hCG-stimulated cAMP production are summarized in Table 1 and Fig. 3. Replacement of Asp564 with the small hydrophobic residues Ala or Val resulted in similar increases in basal receptor activity (2.8-fold for D564A and 2.7-fold for D564V; Fig. 3A).

In Fig. 3B, the consequences of replacing Asp564 in the LHR with either Glu or Asn are compared. This experimental approach has been useful in understanding the mechanistic roles that anionic side chains play in conformational signaling (Cohen *et al.*, 1993; Arnis *et al.*, 1994; Kosugi *et al.*, 1996; Spudich and Lanyi, 1996; Wang *et al.*, 1993). Substitution with Glu (D564E) simply extends the ionizable carboxylate side chain of Asp by one methylene group. This conservative modification was found to have no effect on the basal level of cAMP accumulation. In contrast, substitution of Asp564 with the similarly sized but uncharged Asn residue (D564N) produced a 3.1-fold increase in basal cAMP levels. These results are the exact opposite of those obtained when Glu and Asn were substituted for Asp578 (Kosugi *et al.*, 1996), and suggest that in the case of Asp564 it is the deprotonated (nega-

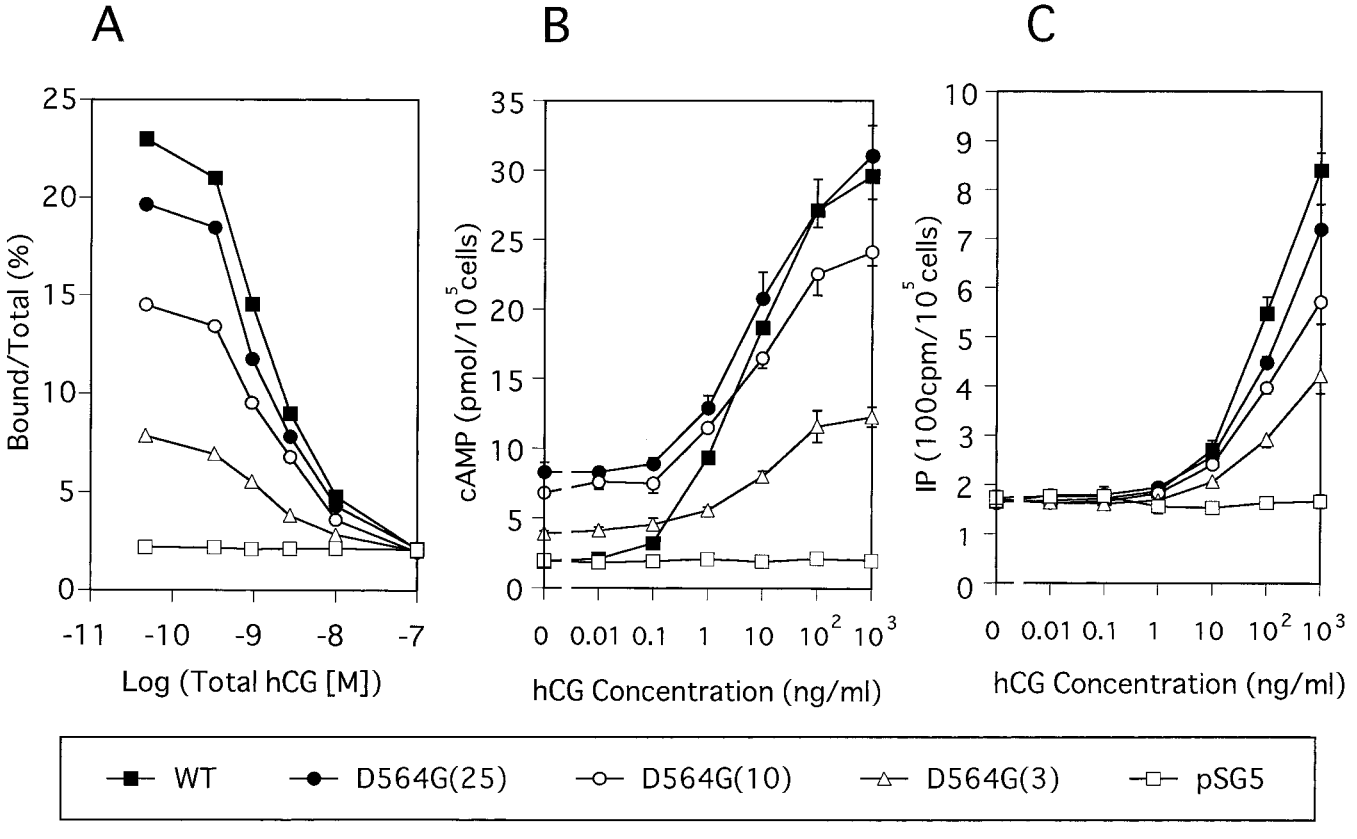

Fig. 2. Radiolabeled hCG binding (A), basal and hCG-stimulated cyclic AMP (B), and IP production (C) in COS-7 cells transfected with DNA for the WT human LHR (25 μg), the D564G LHR mutant (25, 10, or 3 μg), or pSG5 vector alone (25 μg). Results are from a single experiment performed in duplicate (binding) or triplicate (cAMP and IP). Binding displacement curves were analyzed with the program LIGAND (Munson and Rodbard, 1980). Cells transfected with 25, 10, or 3 μg of D564G DNA had binding B_{max} values that were 82, 63, and 27% of the WT B_{max}, respectively, and exhibited 4.4-, 3.6-, and 2.0-fold increases in basal cAMP accumulation. The D564G mutation had no significant effect on LHR affinity for hCG (K_d = 1.1–1.4 nM) and did not cause an increase in basal IP production.

tively charged) form that is important for maintaining the inactive state of the LHR.

Leu and Phe, two residues with large hydrophobic side chains that lack the ability to form a hydrogen bond or electrostatic bond, also caused constitutive activation of the LHR when placed at position 564, with increases in cAMP production of 5.0- and 4.1-fold, respectively (Fig. 3C, Table 1). All of the mutants retained the ability to respond to hCG, but the maximal cAMP responses of those with hydrophobic substituents were only 60–85% of the WT response, and this was not clearly attributable to decreased surface expression (Fig. 3, Table 1). The D564V, D564L, and D564F mutants also exhibited 2–3-fold increases in EC_{50} values. Only the D564L mutant caused constitutive activation of the IP pathway, and the degree of stimulation was small (1.5-fold over WT basal; Table 1).

Mutational additivity. To understand whether Asp564 and Asp578 might play a cooperative role in maintaining the inactive LHR conformation, we combined D564N with each of three mutations known to cause activation of basal cAMP, D578G (4.5-fold), D578S (4.9-fold), and D578Y (7.9-fold), and tested the behavior of the WT, single, and double-mutant LHR constructs in parallel (Table 1; Fig. 4). The high constitutive activities of D564N/D578G (6.7-fold) and D564N/D578S (9.2-fold) indicated that the effects of these paired mutations on cAMP signaling were additive. In contrast, the activity of the D564N/D578Y double mutant (8.5-fold) was only slightly greater than that of the D578Y LHR ($p < 0.05$,

TABLE 1
Summary of activities of mutants involving Asp564 in the human LHR

Mutant	hCG binding		cAMP production			IP production	
	K_d[a]	B_{max} (%WT)[b]	Basal[b]	+1 μg/ml hCG[b]	EC_{50}[a]	Basal[b]	+1 μg/ml hCG[b]
	nM	*%*			*ng/ml*		
WT	1.6(1.0–2.4)	100[c]	1[d]	13.34±0.89	6.1(4.2–8.8)	1[e]	4.77±0.29
D564G	1.0(0.6–1.7)	79±10	3.76±0.33	13.46±1.44	7.7(5.4–10.9)	0.97±0.08	4.13±0.34
D564A	1.1(0.7–1.6)	127±14	2.80±0.80	9.79±1.02	8.8(639–11.1)	1.09±0.07	6.18±1.02
D564V	1.3(0.5–3.0)	73±17	2.69±0.61	9.63±2.65	14.2(12.1–16.7)	1.07±0.10	4.11±1.06
D564E	2.8(2.4–3.3)	146±5	0.99±0.04	16.84±1.30	6.4(4.6–8.9)	1.06±0.04	6.39±0.66
D564N	1.2(1.0–1.4)	94±10	3.14±0.25	16.16±1.17	4.1(2.6–6.5)	1.09±0.07	7.35±0.45
D564L	1.0(0.9–1.2)	70±17	4.96±1.17	8.02±1.77	19.9(15.7–25.3)	1.48±0.13	4.12±0.60
D564F	1.3(1.0–1.8)	80±6	4.09±0.99	11.20±1.36	16.6(10.0–27.5)	1.15±0.19	3.24±0.24
D578G[f]	1.3(0.8–2.3)	81±32	4.53±0.35	13.80±0.21	10.9(9.1–13.0)	1.02±0.05	3.83±0.31
D578S[f]	1.1(0.7–1.8)	111±35	4.91±0.19	15.46±1.41	3.7(2.6–5.0)	1.10±0.07	6.40±0.47
D578Y[f]	1.0(0.4–2.6)	166±17	7.93±0.53	17.91±1.46	10.3(5.9–18.1)	1.89±0.04	6.96±0.34
D564N/D578G	1.8(0.8–5.4)	77±10	6.72±0.46	13.84±0.76	10.9(6.9–17.2)	1.87±0.35	4.26±0.66
D564N/D578S	1.1(0.6–1.9)	71±22	9.15±0.43	14.10±0.64	7.8(5.5–11.0)	2.15±0.51	5.51±1.42
D564N/D578Y	1.6(1.0–2.6)	101±15	8.49±0.57	11.13±0.64	ND[g]	2.40±0.15	4.03±0.27

[a] Geometric mean (95% confidence limit) of at least three experiments.
[b] Mean ± S.E. of at least three experiments.
[c] WT B_{max} averaged 4.2±0.7 × 10^4 receptors/cell.
[d] WT basal cAMP level averaged 1.56±0.36 pmol/10^5 cells.
[e] WT basal IP level averaged 127±26 cpm/10^5 cells.
[f] Data from three to four experiments in which corresponding double mutant construct was studied in parallel.
[g] Nondetectable.

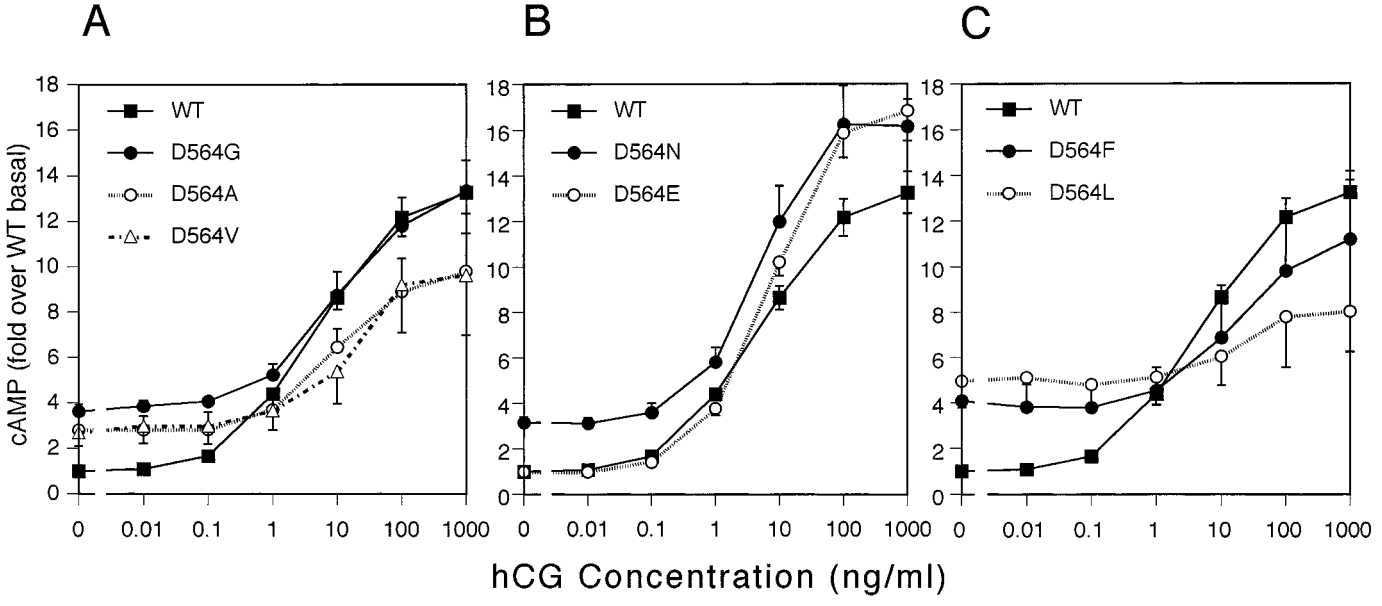

Fig. 3. Cyclic AMP production in COS-7 cells transfected with Asp564 mutant and WT human LHR DNA. Data are mean ± standard error of at least three independent experiments. The WT basal was 1.55 ± 0.35 pmol/10^5 cells.

paired *t* test). Surface expression of all three double-mutant receptors was somewhat lower than that of the single mutants from which they were derived (Table 1), indicating that increased basal activity was an intrinsic property of the altered proteins. The double-mutant receptors had K_d values similar to that of WT, and all responded to hCG, but the maximal cAMP response of D564N/D578Y was significantly decreased (Table 1; Fig. 4C).

Although the D564N, D578G, and D578S mutations had no effect on agonist-independent production of IPs by themselves, the double-mutant receptors D564N/D578G (1.9-fold) and D564N/D578S (2.2-fold) were found to be constitutively activated (Table 1). D578Y alone caused a 1.9-fold increase in basal activity, but the activity of the D564N/D578Y double-mutant (2.4-fold) was even greater ($p < 0.05$, paired *t*-test).

Discussion

In a previous study of the LHR we compared the effects of substituting seven different amino acids for the conserved Asp578 residue in TM6 (Kosugi *et al.*, 1996). Gly, Ser, Glu, Leu, Tyr and Phe caused constitutive activation of cAMP signaling, but Asn did not. Those data suggested that it is the ability of the Asp578 side chain to serve as a properly positioned H-bond participant, rather than its negative charge, that is important for stabilizing the inactive state of the LHR. A rhodopsin-based model of the human LHR places Asp578 near the middle of TM6, oriented toward TM7 (Baldwin *et al.*, 1997; Lin *et al.*, 1997). We hypothesize that H-bonds between Asp578 and residues in TM7 are critical for maintaining the inactive conformation, and that loss or weakening of these interactions increases the proportion of receptor molecules that undergo conformational change in the absence of agonist (Lin *et al.*, 1997).

In the present set of experiments we examined the role of Asp564, another conserved Asp residue that is the site of a naturally occurring activating mutation in the LHR (D564G). Substitution with Gly, Ala, Val, Leu, and Phe produced constitutive activation, but in this case the neutral Asn residue was also activating, and Glu was the sole amino acid replacement that preserved WT basal activity. The fact that only those receptors with an anionic side chain at position 564 were silent in the absence of hormone indicates that a negative charge at this site may be important for stabilizing the inactive LHR conformation.

Bacteriorhodopsin, sensory rhodopsin, and visual opsins are topologically similar, heptahelical transmembrane proteins that become activated in response to light. The mechanism by which they undergo conformational change has been shown to involve dynamic shifts in the protonation state of key anionic side chains (Cohen *et al.*, 1993; Arnis *et al.*, 1994; Spudich and Lanyi, 1996) and a rearrangement of helical packing (Farrens *et al.*, 1996; Spudich and Lanyi, 1996). Replacing anionic Asp and Glu residues with their neutral counterparts (Asn and Gln, respectively) has been used to simulate the effects that local proton transfers have on protein function. For example, a negative charge on a highly conserved residue at the cytoplasmic end of TM3 in rhodopsin (Glu134) is important for stabilizing the inactive receptor state, and G protein binding is favored when the side chain becomes protonated during photoactivation (Cohen *et al.*, 1993; Arnis *et al.*, 1994). A mutant rhodopsin with E134Q has been shown to exhibit mild constitutive activity in the absence of light (Cohen *et al.*, 1993; Arnis *et al.*, 1994).

Recent mutagenesis and molecular dynamics studies of the corresponding Asp142 residue in TM3 of the α_{1B}-adrenergic receptor (α_{1B}-AR) suggest that receptor activation may involve the ability of this side chain to translocate from the cytoplasmic water to a less polar environment as a consequence of protonation, perhaps helping drive larger scale movements of TM3 (Scheer *et al.*, 1997). A mutation of the equivalent residue in the rat LHR (E441Q) does not seem to be activating, however (Wang *et al.*, 1993), and it remains to be seen how generalizable this effect will be among different GPCRs.

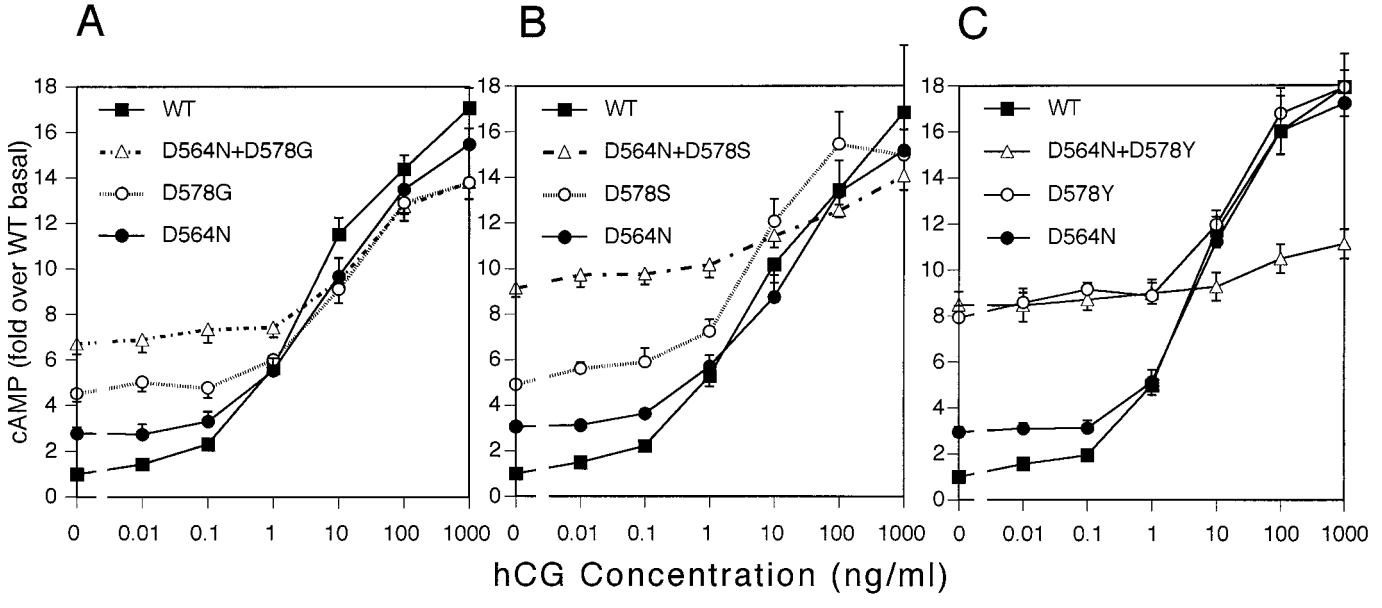

Fig. 4. cAMP production in COS-7 cells transfected with single mutant, double mutant, and WT human LHR DNA. Data are mean ± standard error of three or four independent experiments in which D564N, a D578 mutant, the corresponding D564/D578 double mutant construct, and WT DNA were transfected and analyzed in parallel. In these experiments the WT basal was 1.50 ± 0.27 pmol/10^5 cells.

Evidence for rigid body movement of another helix, TM6, has been obtained for light-activated bacteriorhodopsin (Spudich and Lanyi, 1996) and rhodopsin (Farrens *et al.*, 1996). In both cases it seems that the cytoplasmic end of TM6 tilts away from the central hydrophilic pore. For rhodopsin and other GPCRs this movement may permit key domains, including the amino- and carboxyl-terminal ends of i3, to bind and activate G proteins (Probst *et al.*, 1992; Baldwin *et al.*, 1997. Recent studies of spin-labeled rhodopsin indicate that the amino- and carboxyl-terminal portions of i3 exist as cytoplasmic α-helical extensions of TM5 and TM6, respectively (Altenbach *et al.*, 1996). Glu247, the rhodopsin residue that corresponds to Asp564 in the LHR, is predicted to lie in the aqueous phase, on the inward face of the TM6 extension, and to be engaged in immobilizing tertiary interactions within the protein interior. It is within the segment that is predicted to undergo rigid body movement upon photoactivation (Farrens *et al.*, 1996; Altenbach *et al.*, 1996).

Alterations in TM6 packing may occur naturally as the result of hormone binding, or as the result of activating amino acid substitutions. Activating mutations in TM6 or at the i3-TM6 junction have been discovered or created in a wide variety of GPCRs other than the LHR, including the α_{1B}-AR and other adrenergic receptors (Lefkowitz *et al.*, 1993), the thyrotropin receptor (Parma *et al.*, 1993; Kosugi *et al.*, 1993; Tonacchera *et al.*, 1996), muscarinic receptors (Högger *et al.*, 1995, Spalding *et al.*, 1995; Liu *et al.*, 1996), the platelet-activating factor receptor (Parent *et al.*, 1996), the receptor for parathyroid hormone and parathyroid hormone-related peptide (Schipani *et al.*, 1996), and yeast pheromone receptors (Konopka *et al.*, 1996). The ability of some of these substitutions to drive receptor-G protein coupling has been attributed to changes in charge (Parent *et al.*, 1996) or the relative positioning (Liu *et al.*, 1996) of residues at the i3-TM6 junction.

Some i3-TM6 GPCR mutations involve the anionic residue that corresponds to Asp564 in the LHR. For example, D619G was one of the first somatic thyrotropin receptor mutations found to cause hyperfunctional thyroid adenomas (Parma *et al.*, 1993). A mutation encoding D567G in the follitropin receptor was reported in one male with gonadotropin-independent spermatogenesis (Gromoll *et al.*, 1996); the mildly activating phenotype of this mutant receptor (1.5-fold increase in basal cAMP) was not replicated by others, however (Kudo *et al.*, 1996). An engineered E360A mutation of the m1 muscarinic receptor was found to cause an increase in basal IP production and an increase in agonist affinity (Högger *et al.*, 1995). The residue corresponding to Asp564 in the human β_2-AR is Glu268, and its replacement with Gly produces a 4-fold increase in agonist affinity; these studies were conducted in an expression system where constitutive activity was not directly assessed (O'Dowd *et al.*, 1988).

The studies we have performed on position 564 in the LHR may help reveal the structural role that this residue plays in regulating the conversion between inactive and active receptor conformations. If one assumes that the carboxyl-terminal portion of i3 exists as an α-helical extension of TM6 in the LHR, as it does in rhodopsin, then Asp564 lies just below, and on the same helical face, as most of the other residues that are sites of activating mutations in testotoxicosis (Fig. 1) (Baldwin *et al.*, 1997; Lin *et al.*, 1997). The fact that all the LHR mutants with uncharged residues at position 564 spontaneously undergo transition to the activated state suggests that the Asp side chain carboxyl group is deprotonated in the inactive state, and becomes protonated as the result of agonist-induced activation. Protonation might serve to eliminate a constraining intramolecular electrostatic bridge, alter local secondary structure, or permit a new packing interaction that favors G protein binding. One possibility is that the protonated form of Asp564 needs to become buried in the hydrophobic membrane bilayer (Scheer *et al.*, 1997; Smith *et al.*, 1996) for the LHR to assume its fully activated and coupled conformation, perhaps by facilitating a tilting movement of TM6.

The effects of side chain size at position 564 are apparently less important than they are at position 578 in the LHR (Kosugi *et al.*, 1996). For example, the absence of basal activity in the D564E mutant indicates that side chain length is not critical for maintaining the inactive state; if Asp564 participates in a stabilizing salt bridge with a residue in another loop or helix, it must be one that can accommodate the insertion of an extra methylene group. Unlike position 578, where two bulky aromatic substitutions were found to be especially activating, there is no obvious correlation between physicochemical properties of the residues placed at position 564 and the level of constitutive activity. The Gly, Asn, and Glu mutant LHRs exhibit normal agonist-dependent signaling, but the Ala, Val, Phe, and Leu mutants have mildly impaired responses to hCG, as evidenced by diminished maximal response and/or slightly increased EC_{50} values. These data suggest that the polarity of the side chain at position 564 may also play a minor role in stabilizing the fully activated conformation of the LHR or in G protein binding (Baldwin *et al.*, 1997).

Determining whether the effects of multiple mutations within a molecule sum additively or nonadditively has been used to analyze the structure, stability, activity, and binding affinity of a variety of different proteins (Mildvan *et al.*, 1992; LiCata and Ackers, 1995), including several GPCRs (Cohen *et al.*, 1993; Sealfon *et al.*, 1995; Han *et al.*, 1996; Hwa *et al.*, 1997). Lack of simple additivity between mutation sites in a protein is usually interpreted to indicate direct or indirect contact between the two residues, and cases of partial additivity may occur when one mutation has a long-range effect on the environment of the second site. Synergistic (superadditive) effects of activating mutations have been demonstrated for rhodopsin (Cohen *et al.*, 1993; Han *et al.*, 1996) and for the α_{1B}-AR (Hwa *et al.*, 1997).

The degree to which the double-mutant receptors are constitutively activated may provide insight into the normal mechanisms by which the LHR assumes its active conformation. Combining an activating mutation in TM6 of the LHR (D578G or M571I) with one in TM2 (M398T) was originally reported to have a nonadditive effect on basal LHR activity (Kraaij *et al.*, 1995), but interpretation of those experiments did not include quantitation of receptor number. When the same data are corrected for low receptor expression it seems that one of the two double mutants (D578G/M398T) does demonstrate additivity (Kraaij, 1996). We have recently found that the combination of certain activating mutations at D578 and N619 in TM7 can restore normal receptor basal activity (Kosugi *et al.*, 1997), supporting the concept that these two residues are normally in direct H-bond contact (Lin *et al.*, 1997; Baldwin *et al.*, 1997).

Our current observation that combining D564N with either D578G or D578S has an additive effect on basal cAMP accumulation and a synergistic effect on basal IP production suggests that these substitutions may mimic distinct aspects of the LHR activation process. One simplistic possibility is that a change in charge distribution near the i3-TM6 junction and a rearrangement of TM6 packing both occur as the result of hormone binding, and that mutations that independently promote these processes are able to produce additive effects.

For D578Y, the additional effect of removing the negative charge at position 564 on basal receptor activity was found to be small (Table 1, Fig. 4C). Bulky substituents at position 578 are predicted to elicit a more drastic change in the orientation of TM6 than either Gly or Ser (Kosugi *et al.*, 1996; Lin *et al.*, 1997), and lack of simple additivity might be due to a Tyr-induced structural perturbation that influences the environment of residue 564. If the single D578Y mutant were to increase the pK_a value of Asp564 by shifting the cytoplasmic end of TM6 into a more hydrophobic environment, then the additional effect of mutating Asp564 to a neutral Asn would be minimal. The concept that the effects of a mutation at position 564 in the LHR may be coupled in some way to the packing configuration of TM6 receives support from recent studies of chimeric glycoprotein hormone receptors (Kudo *et al.*, 1996).

For the human LHR, the only mutant receptors that cause agonist-independent activation of the IP pathway are those that have also been found to elicit strong constitutive activation of the cAMP pathway, namely D578L, D578Y, D578F, D564L, and the three double mutants reported here (Kosugi *et al.*, 1996) (Table 1). This is consistent with the fact that the LHR couples less efficiently to the IP signaling pathway (Gudermann *et al.*, 1992; Kosugi *et al.*, 1996). Although there is some evidence that glycoprotein hormone receptors may utilize different conformations to activate the two pathways (Tonachera *et al.*, 1996; Gilchrist *et al.*, 1996), no glycoprotein hormone receptor mutation has been reported that results in constitutive activation of the IP pathway without substantial activation of cAMP pathway as well.

In conclusion, our mutagenesis data on the human LHR suggest that the type of stabilization provided by the Asp564 side chain at the i3-TM6 junction (electrostatic) is different from that provided by the Asp578 side chain near the middle of TM6 (interhelical H-bonding). We hypothesize that agonist-dependent activation of the LHR involves protonation of Asp564 and a rearrangement in the packing of TM6, and that mutations that partially mimic or promote these processes are able to trigger G protein coupling. Defining the distinct roles played by electrostatic, H-bond, and hydrophobic interactions is important for understanding the mechanism of activation of the LHR and of other heptahelical membrane proteins that undergo conformational change.

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