Hydrophobic Amino Acid in the i2 Loop Plays a Key Role in Receptor-G Protein Coupling*

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Signal transduction of the heptahelical G protein-coupled receptors (GPCRs) involves multiple receptor domains, but a universal consensus domain for coupling has not yet been defined. Alanine mutagenesis scanning was performed on the intracellular loops and the COOH tail of the human muscarinic cholinergic receptor (Hm1) to identify coupling domains. Stimulation of phosphatidylinositol (PI) turnover was determined after transfection of the alanine mutants into U293 human embryonic kidney cells. Alanine substitutions in four regions (loops i1, i2, and NH2 and COOH junctions of i3) impaired coupling efficiency by ~50% or more, but the strongest reduction (>80%) resulted from alanine replacement of a single amino acid, leucine 131. This residue is located in the middle of the second intracellular loop (i2), within the highly conserved GPCR motif (DRYXXV(L)XXP(L)). The position equivalent to Leu-131 in Hm1 contains a bulky hydrophobic amino acid (L, I, V, M, or F) in nearly all cloned GPCRs. Substitution of Leu-131 with polar amino acids (aspartate and asparagine) also resulted in strongly defective coupling, whereas phenylalanine (found in the equivalent position in the β2 adrenoceptor) can replace leucine without losing PI coupling ability of Hm1. Alanine substitution of the corresponding amino acid in the i3 receptor (L174A) also inhibited agonist-stimulated PI turnover, while replacing Phe-139 with alanine in the β2 adrenoceptor suppressed stimulation of adenylyl cyclase. We propose that a bulky hydrophobic amino acid in the middle of the i2 loop serves as a general site relevant to G protein coupling, whereas coupling selectivity is governed by other receptor domains.

The Hm1 muscarinic cholinergic receptor is a member of the large family of G protein-coupled receptors (GPCRs). The structure of GPCRs is thought to consist of seven transmembrane helices, with three intracellular loops (i1-i3) and the cytoplasmic COOH tail (1). Studies with mutagenesis (2, 3), receptor-derived peptides (4-6), and receptor antibodies (7) have shown that multiple domains of the intracellular receptor portion contribute to functional G protein coupling. However, a universal consensus domain has yet to be identified, and suggested coupling sites show greatly divergent sequences such as the highly variable i3 loop of catechol and peptide neurotransmitter receptors. On the basis of previously proposed coupling domains, we introduced mutations into the i3 loop of Hm1 expected to uncouple the receptor from its G protein (8), but the ability of the mutant receptors to stimulate phosphatidylinositol (PI) turnover was barely affected. This result prompted us to undertake alanine mutagenesis scanning of the intracellular surface of Hm1. Several domains were identified where alanine substitutions interfered with carbachol-stimulated coupling to PI turnover, and a conserved lipophilic residue in loop i2 (Leu-131 of Hm1) was found to play a major role in receptor coupling.

EXPERIMENTAL PROCEDURES

Materials—[^3]HINMS (specific activity of 80 Ci/mmol) and [^3H]CGP12177 (specific activity of 46 Ci/mmol) were obtained from American Radiolabeled Chemicals, Inc. All other reagents were of analytical grade quality.

Construction of Vectors Expressing Hm1, Hm3, and β2 Point Mutants—The construction of wild-type Hm1 and Hm3 in vector pSG5 was described previously (9-11). The hamster β2 adrenoceptor, in pCDNANeo and in Bluescript SK+, was obtained from C. D. Strader (12). Mutagenesis was performed with the BlueScript vector before transferring the mutated β2 gene, cut with XhoI, back to pCDNANeo. All point mutations were introduced using the “unique site elimination” method (Transformer™ site-directed mutagenesis kit, Clontech) (13). All mutants were analyzed by restriction mapping and sequencing of the mutated region before use.

Transfection of Human Embryonic Kidney Cells (U293)—The cells were transfected with the use of the calcium precipitation method as described before (9, 10). Transient expression yields were ~900 fmol/mg protein for Hm1, whereas some Hm1 mutants yielded considerably lower expression. Maximal stimulation of PI turnover by 1 mM carbachol appears to be depressed if the expression level drops below 250 fmol/mg protein; therefore, receptor expression was always measured in parallel with PI turnover. Experiments that yielded less than 250 fmol/mg protein were excluded from the data analysis. Expression of Hm3 and β2 wild-type and their respective mutant receptors gave comparable and very high yields (data not shown).

Receptor Binding Assays—Receptor expression of Hm1, Hm3 and their respective mutants was determined in intact cell monolayers as described previously (9, 10) using 2 nM [^3H]NMS. Binding competition curves with carbachol, to determine the carbachol IC50 binding value, were also carried out in intact cells using 0.1 nM [^3H]NMS. Receptor expression of the β2 receptor and its mutant was determined in cell homogenates using 3 nM CGP12177 (14) as described. Competition curves with isoproterenol were obtained with 0.5 nM CGP12177.

PI Turnover—PI turnover was measured after labeling the cells with [^3H]myoinositol (24 h—9-11). For the assay of inositol monophosphate, which accounts for most of the inositol phosphate [^3H]activities in the presence of 1 mM LiCl, six-well cell culture dishes (well diameter of 3.5 cm) were used. Results were expressed as percent of total [^3H]activity, and the percent values were compared between carbachol-treated and untreated cells.

cAMP Assay—Isoproterenol dose response for AMP production was measured in the absence of a phosphodiesterase inhibitor. Intact attached monolayer cells were rinsed once with 1 ml of serum-free Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES. Serum-free medium was added to each well (final volume 300 μl), followed by 10 μl of the appropriate concentration of isoproterenol. The cells were incubated at 37 °C for 15 min. The reaction was terminated by the addition of 100 μl of 0.4 M HCl. The cells were then incubated at 95 °C for 5–10 min, scraped off the wells, transferred into 1.5-ml polypropylene microcentrifuge tubes, and centrifuged at 4 °C for 5 min. The supernatant was collected and assayed for cAMP content with a competitive protein binding assay kit (Amersham Corp.). Protein content was determined by the Bradford method using the U. S. Biochemical Corp. reagent; it ranged from 0.25 to 0.3 mg/well. The data were fitted to the equation E = (E∞max × L)/(E∞max + L), where E is the stimulation of...
cAMP production over control values and $E_{max}$, the maximum stimulation. The results were fitted with the Minim 1.2 program (9–11), after deletion of the data at $10^{-5}$ m isoproterenol, which may have resulted in lower cAMP levels by $i^2$ stimulation of $G_i$ (15).

RESULTS AND DISCUSSION

Receptor portions of Hml shown by deletion mutations not to participate in coupling (3, 10) were excluded from the alanine scanning analysis (Fig. 1, arrows). Additionally, mutations of the following residues of Hml have been previously shown by us not to affect coupling significantly: Glu-214, Thr-215, Glu-216, Lys-359, Lys-361, Ser-366, Thr-428 (Fig. 1, solid circles) (8, 10). Of the remaining intracellular residues, both lipophilic and polar/charged amino acids were selected to cover receptor regions with possible involvement in coupling. Assuming that each coupling domain may entail several amino acids, this strategy was felt to cover most or all potential coupling sites. A

Nine multiple alanine point mutants and one COOH tail truncation mutant were constructed (Fig. 1). Three out of 10 mutants (mutations 3, 9, and 10; Fig. 1) did not yield productive tracer binding; therefore, carbachol-stimulated PI turnover was assayed for only seven mutants. Mutant 5 was modestly deficient in coupling to PI turnover; lower receptor expression relative to the wild-type in each individual experiment may have contributed to this result (Table I). Mutants 6 (W209A/I211A/Y212A) and 7 (E360A/K362A/T366A) yielded tracer binding only slightly lower than the wild type but were defective in coupling (Table I). Cheung et al. (3) previously demonstrated that specific hydrophobic, but not hydrophilic residues, in the NH$_2$-terminal junction of the i3 loop of the $p_2$ adrenoceptor are involved in G protein coupling. The coupling-deficient mutant 6 also implicates lipophilic residues of the NH$_2$-terminal i3 junction in coupling of Hml. Similarly, mutant 7 involves changes in the COOH-terminal i3 loop junction, which has been implicated in coupling (2, 3). These results support a functional coupling role for the COOH- and NH$_2$-terminal junctions of the i3 loop. Furthermore, mutant 1 (K51A/N52A/N54A) displayed impaired coupling, also implicating the i1 loop in this process. The receptor expression yield in each of the reported cases in Table I was above 250 fmol/mg protein, which we consider a threshold for allowing near-maximal stimulation of PI turnover by Hml, on the basis of numerous previous transfection experiments with Hml and its mutants (8–11). However, in the absence of an accurate molecular model at atomic resolution, the interpretation of partially defective coupling of mutant receptors is difficult because the loop and COOH tail junctions must form a tightly packed structure that responds to agonist-induced activation.

Only mutant 4 (L131A/Y133A/K136A, located in the middle of the i2 loop) was profoundly deficient in mediating PI turnover (Table I). Since this mutant contains three alanine point mutations, we constructed the individual alanine point mutants. Unexpectedly, mutant L131A was again strongly defective, while receptor expression was similar to that of the wild-type. In contrast, Y133A and K136A were as efficient as the

![Fig. 1. Deduced amino acid sequence of Hml.](image)

The locations of nine multiple point mutations (1–9) and the COOH tail truncation (10) were selected to avoid any overlap with previous mutations. Point mutations previously shown not to affect significantly G protein coupling (8, 10) are shown as filled circles, whereas the largest deletions of the i3 loop that still yielded full coupling efficiency (9, 10) are shown by the arrows.

TABLE I

Effect of point mutations on receptor expression and coupling efficiency to phosphatidylinositol turnover:

Stimulation of PI turnover by 1 mM carbachol is given as a percentage of the wild-type receptor stimulation, after subtraction of background stimulation in nontransfected cells (9–11). Receptor expression was measured with 2 nM [3H]N-methylscopolamine ([3H]NMS) using intact cells (9–11). Relative stimulation of PI turnover is shown for each single experiment where receptor yield exceeded 250 fmol/mg protein.

| PHNMS | Stimulation of [3H]PI release | % of Hml wild-type stimulation |
|-------|-------------------------------|-------------------------------|
| Hml wild-type | 920 ± 236 | (n = 19) | 100 ± 7 | (n = 26) |
| Mutants |
| 1. K51A/V52A/T54A | 960 ± 117 | (n = 6) | 52 ± 11 | (n = 4) |
| 2. K51A/T58A/V59A | 906 ± 306 | (n = 3) | 99 ± 34 | (n = 4) |
| 3. Y124A/S128A/V127A/T128A | Not detectable | (n = 3) | 100 ± 11 | (n = 4) |
| P125A | 888 ± 37 | (n = 6) | 48 ± 6 | (n = 6) |
| V127A | 443 ± 32 | (n = 5) | 96 ± 29 | (n = 8) |
| P130A | 1058 ± 912 | (n = 3) | 15 ± 7 | (n = 4) |
| L131A | 958 ± 47 | (n = 4) | 15 ± 5 | (n = 6) |
| L131N | 919 ± 93 | (n = 5) | 15 ± 2 | (n = 4) |
| L131D | 798 ± 48 | (n = 5) | 10 ± 12 | (n = 4) |
| L131F | 1001 ± 70 | (n = 4) | 94 ± 7 | (n = 40) |
| L131M | 508 ± 43 | (n = 6) | 60 ± 14 | (n = 4) |
| Y133A | 309 ± 47 | (n = 3) | 71 ± 24 | (n = 5) |
| K136A | 678 ± 54 | (n = 4) | 94 ± 7 | (n = 4) |
| 5. R137A/T138A/R140A | 392 ± 82 | (n = 3) | 59 ± 26 | (n = 5) |
| 6. W209A/I211A/Y212A | 733 ± 41 | (n = 5) | 34 ± 12 | (n = 7) |
| 7. E360A/K362A/T366A | 542 ± 28 | (n = 5) | 39 ± 6 | (n = 7) |
| 8. K429A/R428A | 562 ± 29 | (n = 5) | 89 ± 6 | (n = 5) |
| 9. P425A/D427A/F429A | Not detectable | (n = 5) |
| 10. R430 trunc | Not detectable | (n = 5) |

a Statistically significant compared with wild-type, $p < 0.0001$ (one factorial ANOVA and Fisher PLSD).

b Receptor yields for Tyr-133 and mutants 4 and 5 were consistently severalfold lower than those of the Hml wild-type in individual experiments.
wild-type receptor in stimulating PI turnover. These results suggest that Leu-131 represents a novel site with relevance to G protein coupling. Replacement of Tyr-133 with alanine lowered receptor expression significantly, indicating a possible role of this residue in internal folding. Indeed, only a single experiment yielded receptor expression above 250 fmol/mg protein (Table I), whereas the yield was below 150 fmol/mg protein in additional experiments, preventing quantitative analysis of maximal PI turnover. Hence, impaired coupling but normal expression of L131A suggests that this residue interact directly with the G protein, rather than affecting internal folding.

A dose-response curve with carbachol shows the profound effect of the L131A substitution in Hm1 on PI turnover (Fig. 2A). The binding affinity of the Hm1 mutant L131A to carbachol (IC_{50} = 0.28 ± 0.03 mM) was largely unchanged from that of the wild-type receptor (IC_{50} = 0.34 ± 0.03 mM), indicating that loss of coupling efficiency was not caused by a change in agonist affinity.

Sequence comparison among 70 cloned mammalian GPCRs (16) indicates that a lipophilic amino acid is well conserved at the site corresponding to Leu-131 in Hm1. A few selected sequences are shown in Table II. This site is located at the 3' end of a highly conserved 12 loop motif with the following most common residues: DRYXXV(1)XXPL, where X is any amino acid. Substitution of aspartate with asparagine in the DRY motif of the muscarinic m1 receptor resulted in reduced G protein coupling (17). The residue immediately adjacent to DRY is somewhat conserved, consisting of lipophilic aromatic or aliphatic amino acids in many GPCR sequences. However, substitution of Phe-125 with alanine had no effect on Hm1 expression and coupling (Table I). In contrast, position Val-127 is very highly conserved and contains either valine or isoleucine in 67 out of the 70 mammalian GPCRs listed in Ref. 16. Alanine substitution of Val-127 in Hm1 indeed caused a significant loss in coupling efficiency, although the effect was smaller than for the L131A mutant (Table I). Mutant V127A also showed some impairment of overall receptor expression. These results indicate that position Val-127 contributes to the 11 loop coupling domain either directly or indirectly, by affecting Hm1/12 folding. One amino acid 5' to Leu-131 is predominantly occupied by proline (in 54 out of 70 sequences) (16), which has been shown previously to play a small but measurable role in β2 adrenoceptor coupling (18). Substituting Pro-130 with alanine (Hm1 mutant P131A) did not affect carbachol-stimulated PI turnover relative to wild-type Hm1 (Table I). Alanine residues are found in a few receptors at the position equivalent to Pro-130, such as the α2 adrenoceptor subtypes and the glycoprotein hormone receptors (16), suggesting that alanine substitution is permissive for G protein coupling.

The Leu-131 site contains bulky lipophilic amino acids in 64 out of 70 mammalian GPCR sequences (16); leucine occurs 27 times, while isoleucine (12 times), valine (4 times), methionine (11 times), and phenylalanine (10 times) are also found. We therefore hypothesized that a bulky hydrophobic amino acid is required in the well defined position Leu-131. Indeed, substitution of Leu-131 with phenylalanine (e.g. β2 adrenoceptor) yielded a mutant Hm1 receptor with productive PI coupling. Substitution with methionine to give L131M still supported measurable, but clearly reduced PI turnover, whereas point mutants with the polar amino acids aspartate or asparagine were highly deficient (Table I). In each case, overall receptor expression was similar to that of the wild-type receptor. Hence, a lipophilic amino acid in position 131 appears to be required for Hm1 coupling to PI turnover.

Since the muscarinic Hm3 receptor is closely related to the Hm1 receptor (19), the equivalent Leu-174 residue was replaced with alanine. Again, PI coupling was inhibited without strongly affecting receptor expression measured with 2 nM [3H]NMS (Hm3 wild-type, 1974 ± 110 fmol/mg protein; L174A,
1240 ± 288 fmoI/mg protein). A dose-response curve with carbachol shows the profound effect of alanine substitution in Hm3 Leu-174 (Fig. 2B). Because of the low coupling efficiency of the mutants, EC50 values could not be determined.

As both Hm1 and Hm3 couple to G proteins that activate phospholipase C (19), we chose the β2 adrenoceptor to test whether this site is also crucial for coupling to Go, which activates adenyl cyclase. Introduction of the equivalent point mutation F139A caused a significant loss in isoproterenol-induced cAMP accumulation at an equivalent level of β2 receptor expression (wild-type, 4300 fmoI/mg protein; F139A, 4100 fmoI/mg protein). A dose-response curve (Fig. 2C) shows that maximum stimulation of cAMP accumulation is reduced by ~75% and the EC50 is shifted from 1 nM to 7 nM. In contrast, isoproterenol binding curves measured with [3H]CGP12177 indicated only a minimal change of affinity of the mutant receptor (wild-type IC50 3.3 ± 0.3 μM; F139A, 5.3 ± 0.8 μM). Therefore a nonpolar amino acid residue in the middle of the i2 loop, i.e. Phe-139, also plays an important role in signal transduction to adenyl cyclase via Go.

Involvement of the i2 loop in G protein coupling was suggested previously, although a single amino acid had not been identified as a coupling site. A synthetic peptide corresponding to the entire i2 loop of the turkey erythrocyte β1 adrenoceptor suppressed adenyl cyclase activation by 90% (20). Furthermore, transducin prevented the rhodopsin binding of an anti-peptide antibody generated against the i2 loop of rhodopsin (7). Substitution of the entire i2 loop with the the analogous region of the m1 muscarinic receptor has been shown not to affect coupling to adenyl cyclase of the β2 adrenoceptor (21). These results implicated the i2 domain as a coupling site for all G proteins; however, since the i2 loops of m1 and β2 are interchangeable, coupling specificity appears to reside at other receptor domains, e.g. the i3 loop (22). These combined results support the view that receptor-G protein coupling involves multiple domains.

In conclusion, the i2 loop appears to be central to receptor folding, activation, and G protein coupling. The lipophilic amino acid in the middle of i2 (e.g. Leu-131 of m1, Leu-174 of m3, and Phe-139 of β2) in the conserved domain DRYXVIIIXXPL (L = leucine or other lipophilic amino acid) could represent a major coupling site. This site may interact with conserved residues of the COOH-terminal domain of Go subunits, which also contain conserved lipophilic amino acids (23). However, we cannot exclude the possibility that this hydrophobic residue is essential to the internal folding of the i2 loop itself to elicit productive activation. Measuring second messenger activation does not distinguish between receptor-G protein binding and activation, whereas these processes can be readily differentiated for rhodopsin by optical measurement (24). Nevertheless, the results document the importance of the central portion of the i2 loop in G protein coupling within a motif that is highly conserved even among distantly related GPCRs. The crucial relevance of the lipophilic anchor residue in i2 to receptor function is further documented by our recent finding that the Hm1 mutant L131A is also deficient in agonist-induced internalization and down-regulation.2 Hence, this residue may also contribute to the elusive receptor domains mediating cellular trafficking of GPCRs. In a small number of cloned GPCR genes, such as the VIP (25) and secretin (26) receptors, the i2 loop sequence deviates considerably from the motif consisting of DRYXVIIIXXPL. It remains to be determined which role the i2 loop plays in the G protein coupling of these receptors.

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