Identification of a Ligand-dependent Switch within a Muscarinic Receptor*

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G-protein-coupled receptors spontaneously switch between active and inactive conformations. Agonists stabilize the active conformation, whereas antagonists stabilize the inactive conformation. In a systematic search for residues that participate in receptor function, several regions of the m5 muscarinic receptor were randomly mutated and tested for their functional properties. Mutations spanning one face of transmembrane 6 (TM6) were found to induce high levels of receptor activity in the absence of agonists (constitutive activity). The same face of TM6 contained several residues crucial for receptor activation by agonists and one residue identified as a contact site for both agonists and antagonists. In addition, one mutation induced agonist-like responses from the receptor when exposed to classical antagonists. These results suggest that TM6 is a switch that defines the activation state of the receptor, and that ligand interactions with TM6 stabilize the receptor in either an active or an inactive conformation.

Although the primary structures of the five muscarinic receptor subtypes (m1–m5) have been known for a decade (1–4), relatively little is known about either their three-dimensional structures or their activation mechanisms. The muscarinic receptors belong to the family of G-protein-coupled receptors that have significant sequence and functional homology with the visual pigment rhodopsin. There is considerable physical and modeling data to suggest that G-protein-coupled receptors have seven transmembrane domains (TM1–7)1 and these are a-helical (5, 6), but because of difficulties in crystallization, there currently exists only a low resolution model of the three-dimensional structure of these receptors (6). Interactions between muscarinic receptors and G-proteins are believed to be mediated by cytoplasmic loops (i1–i3) linking the transmembrane domains (7, 8). The second internal loop (i2) and a small number of amino acids adjacent to TM5 and TM6 in the N- and C-terminal regions of the third cytoplasmic loop (N-i3 and C-i3) appear to make the largest contribution to receptor-G-protein coupling (9–11). Interactions with ligands are mediated by the transmembrane domains. Both agonist and antagonist ligands have been shown to interact with an aspartate residue in TM3 through their positively charged nitrogen headgroups (12–14), whereas other residues necessary for functional interactions with ligands have been identified in TM2, 5, 6, and 7 (12, 15–18).

A limitation of data collected from classical site-directed mutagenesis is that since relatively few residues have actually been tested, the relative contribution of the individual residues and even the overall domains is difficult to gauge in a broad context. To gain such a broad perspective, it would be necessary to systematically mutate at least a significant proportion of the residues of a receptor and test the relative functional consequences of those mutations. The combination of random mutagenesis with high throughput assays of receptor function make the systematic mutagenesis of a muscarinic receptor feasible. We have been systematically examining the structure-function relationships of receptors by random mutagenesis (9, 11). In the course of these studies, we have found that TM6 plays a vital role in receptor function. Mutations in this region have profound effects on interactions with ligands, and many mutations in TM6 cause elevated activity in the absence of added agonists (constitutive activity).

MATERIALS AND METHODS

Mutagenesis—Random mutations were introduced into the m5 receptor by PCR using a primer having the consensus sequence of the targeted region, but with bases randomly misincorporated at a rate of 11.5% at each position (9, 11). The amino acid sequences of the mutated regions were as follows: TM6, ALLAFITWTPYNMVLVST; C-i3, HMQTKKRRVVLKERAQTLSS; TM5, TITFGTAIAFAVYPSVMITI; N-i3, LCRIYRTETKRTKDLADLQ; i2, DRYFSTIRPLTYPARKRTKRAGIMI.

To construct receptors containing point mutations, a NheI site was introduced at positions Leu-457 and Leu-458 (CTC→CTG to CTC→CTA) without changing the amino acid sequence. Point mutations were introduced by PCR using mutagenic oligonucleotides where the bases encoding Asn-459 and Phe-451 were replaced with an equimolar mixture of all four bases (9). Mutant receptors were screened for their ability to respond to carbachol and only functional receptors were sequenced and subjected to more detailed analysis.

Functional Assays—Functional assays were carried out using receptor selection and amplification technology (R-SAT) (U. S. patent number 5,707,798; see Refs. 9, 19, and 20) This technology is based on the propensity of NIH-3T3 cells to be transformed by muscarinic receptors signaling through the phosphatidylinositol pathway (21). A detailed protocol is given in Ref. 9.

Comparison of Constitutive Activity Levels—For comparing the constitutive activity levels of libraries of receptors mutated in TM6, TM5, i2, C-i3, and N-i3, 6 ng of mutant pCD-m5 was co-transfected with 40 ng of pS1-β-galactosidase into each of six wells of a 96-well plate containing 10,000 NIH-3T3 cells/well. Two days after transfection, cells were exposed in duplicate to no drug, 100 μM carbachol, or 1 μM atropine in

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1 The abbreviations used are: TM, transmembrane domain; PCR, polymerase chain reaction; NMS, N-methyl scopolamine; QNB, quinuclidinyl benzilate; GppNHp, guanyl-5’-yl imidophosphate.
Dulbecco's modified Eagle's medium supplemented with 2% cyto-sf3 (a synthetic supplement which contains no small ligands, Kemp Laboratories). Assays were stopped by lysing cells with detergent, and cell proliferation was quantified by measuring a colorimetric assay. Only mutants having wild-type-like or constitutively activated phenotypes were included in this experiment. 24 TM6, 15 TM5 and 41 i2 mutants having an EC50 for carbachol less than 5-fold greater than the wild-type receptor were considered.

Concentration-Response Experiments—For concentration-response experiments, 200,000 NIH-3T3 cells in a 3.5-cm dish were transfected with 0.5 μg (Table I) or 1 μg (Table II) of recombinant pCD-m5 and pS1-β-galactosidase (Promega). Two days after transfection, the cells were split into 20 wells of a 96-well plate and exposed to different concentrations of ligands for 4 days in Dulbecco's modified Eagle's medium supplemented with 2% cyto-sf3 and 0.5% calf serum. Assays were stopped by lysing cells with detergent, and cell proliferation was quantified by measuring β-galactosidase concentrations using a colorimetric assay. Only mutants having wild-type-like or constitutively activated phenotypes were included in this experiment. 24 TM6, 15 TM5 and 41 i2 mutants having an EC50 for carbachol less than 5-fold greater than the wild-type receptor (Table I) and 16 Ci3 and 15 Ni3 mutants defined as wild-type-like in Refs. 9 and 11 were tested.

Concentration-Response Experiments—For concentration-response experiments, 200,000 NIH-3T3 cells in a 3.5-cm dish were transfected with 0.5 μg (Table I) or 1 μg (Table II) of recombinant pCD-m5 and pS1-β-galactosidase (Promega). Two days after transfection, the cells were split into 20 wells of a 96-well plate and exposed to different concentrations of ligands for 4 days in Dulbecco's modified Eagle's medium supplemented with 2% cyto-sf3 and 0.5% calf serum. Assays were stopped by lysing cells with detergent, and cell proliferation was quantified by measuring β-galactosidase concentrations using a colorimetric assay. To account for minor variations in transfection efficiency, response values were normalized relative to the maximum response of endogenously expressed protonated FP receptors to 100 nm cloprostenol. A receptor-saturating concentration of atropine was included in these assays to suppress constitutive activity of the mutant receptors.

Constitutive Activity—Constitutive activity was measured as receptor activity in the absence of agonists that could be reversed by the antagonist atropine. For the Asn-459 mutants, this measurement was confirmed using benztropine. Maximum response was defined as the difference between the receptor’s maximum response to carbachol and its minimum response to atropine. EC50 denotes the concentration of a ligand that produces a half-maximal response from the receptor in the absence of other ligands. Where the EC50 of atropine could not be determined because of low levels of constitutive activity, receptors were incubated with a fixed concentration of carbachol (2–8 times the EC50 of CCh for that receptor), and varying concentrations of atropine. The resultant atropine IC50 values (concentration inhibiting half of the carbachol-induced activity) were then used to calculate IC50/EC50 values for each receptor using the equation: Kd(atr) = IC50(atr)/1 + [CCh]/[IC50(CCh)], where atr is atropine.

Radioligand Binding Assays—Radioactive binding assays demonstrated that the wild-type and the highly constitutively activated receptor S465Y,T466P were expressed at equal numbers in NIH-3T3 cells (20). Further experiments were carried out as described (15) on membrane preparations derived from COS and TSA cells transfected with representative mutant receptors. Receptor expression levels and [3H]NMS affinities were determined using six concentrations of [3H]NMS. [3H]NMS affinities were (log Kd, nM): wild-type, −9.9 ± 0.1; I447S, −9.6 ± 0.4; N459H, −8.5 ± 0.5; F451L, −10.1 ± 0.4; S465Y,T466P, −9.7 ± 0.0. None of these mutant receptors were found to be significantly overexpressed compared with the wild-type (Bmax, pmol/mg protein) wild-type, 0.8 ± 0.1; I447S, 0.7 ± 0.5; F451L, 0.7 ± 0.3. Bmax for N459H could not be accurately determined because of its low affinity for [3H]NMS. Data for S465Y,T466P are published (20). Carbachol affinities were measured in the presence of 50 μM GppNHp and in competition with 400 pm [3H]NMS, except N459H, which was competed against 2 nM [3H]NMS. Carbachol potencies were: (log IC50) wild-type, −4.4 ± 0.1; I447S, −6.7 ± 0.1; N459H, −4.8 ± 0.5; F451L, −5.2 ± 0.2; S465Y,T466P, −6.1 ± 0.2. Radioligand binding studies could not be conducted on the mutant F451P because of its low affinity for [3H]NMS.

RESULTS AND DISCUSSION

Random Mutagenesis Reveals That Mutations in TM6 Cause High Levels of Constitutive Activity—We have used random mutagenesis to identify functionally important residues in the five regions of the m5 muscarinic receptor indicated in Fig. 1A. Each region of 20–21 amino acids was targeted individually. Mutations were introduced using a PCR protocol that produced an average of five amino acid substitutions per receptor (11). A library of between 500 and 1,200 unique recombinant receptors was constructed for each region to be tested, and the receptors were assayed for functional activity. Functional receptors were identified, sequenced, and characterized pharmacologically.

Out of a library of 1,200 receptors containing mutations in TM6, 34 functional receptors were identified and characterized. It was immediately noticeable that many of these receptors showed high basal activity levels, and that this agonist-independent (constitutive) activity was reversed by antagonists. Fig. 1B shows a comparison of the constitutive activity levels of receptors containing random mutations in TM6 (filled bars) with those containing mutations in TM5, i2, N-i3, and C-i3. The highest levels of constitutive activity and the highest proportion of constitutively activated receptors were seen in library of receptors mutated in TM6. High levels of constitutive activity were also seen in receptors mutated in i2.2

Random Saturation Mutagenesis Identifies Side Chains Essential for Maintaining the Active and Inactive Conformations of the Receptor—The 34 functional receptors obtained from the TM6 mutant receptor library were sequenced, and their pharmacology was evaluated using the agonist carbachol and the
Constitutive activity and maximum response are expressed as a percentage of the maximum response of the wild-type receptor to carbachol. Values represent the mean of at least two determinations, and are rounded to 10% or one significant figure. Functionally conserved residues, which were mutated at these positions, it follows that the receptor tolerated multiple, non-conservative mutations to 14 of the 21 residues in TM6 without severely compromising its ability to be activated by carbachol. These side chains are therefore not essential either for receptor activation by carbachol or for maintaining the overall structure of the receptor. We combined these data with data from an analogous study on the cholerasic receptor (15, 16); however, the effects of mutating residues where mutations were either never seen or were always associated with a greater than 5-fold decrease in carbachol potency. Bold text indicates residues that were never mutated except in receptors showing a greater than 500-fold decrease in carbachol potency. Plain text, Leu-463 was non-conservatively mutated only in receptors showing a greater than 5-fold decrease in carbachol potency. Constitutively activating residues: five residues that were positively identified as sites where point mutations can cause constitutive activity (see Fig. 3, A and B).

| Constitutive activity | Max response | Carbachol EC50 | Atropine EC50 |
|-----------------------|--------------|----------------|---------------|
| 446 LILFA ITW TPYNI MVLVST | 160 | 190 | 1 | 4000 |
| .M... .H... .C... | 90 | 120 | 0.1 | 5 |
| .L... .F... .Y... | 90 | 120 | 0.1 | 10 |
| .L... .L... .Y... | 80 | 130 | 0.2 | 3 |
| .L... .S... .Y... | 70 | 110 | 0.1 | 9 |
| .M... .L... | 70 | 170 | 0.2 | 1 |
| .S... | 60 | 100 | 0.03 | 9 |
| .T... .MV... | 60 | 140 | 0.2 | 7 |
| LV.S... | 50 | 150 | 0.2 | 4 |
| .L... | 40 | 120 | 0.2 | 4 |
| .V... | 30 | 130 | 0.3 | 2 |
| GN... | 30 | 190 | 5 | 50 |
| .L... .P... .F... .S... | 20 | 100 | 0.3 | 7 |
| VM...G | 20 | 90 | 0.4 | 2 |
| .V... | 20 | 120 | 0.4 | 2 |
| .L... A...V... | 20 | 120 | 0.6 | 7 |
| .NV... | 10 | 110 | 1 | 3 |
| .Wild-type | 4 | 100 | 1 | 1 |
| #... | 20 | 40 | 2 | 10 |
| .L... V... | 8 | 120 | 2 | 10 |
| G... .MC... L... | 20 | 110 | 3 | 10 |
| .H... .T... | 5 | 100 | 4 | 1 |
| .M... .F... | 4 | 100 | 5 | a |
| .V... .K... | 6 | 90 | 6 |
| .L... L... | 10 | 80 | 8 | 2 |
| #... | 11 | 60 | 10 |
| .V... | 20 | 120 | 70 |
| .SS... | 5 | 80 | 70 |
| S... | 0 | 130 | 70 |
| T.M...V...S...I... | 8 | 20 | 100 | 3 |
| .V... | 4 | 60 | 200 |
| G... | 9 | 110 | 500 |
| G... .C... | 0 | 100 | 500 |
| V... | 9 | 100 | 1000 |

**TABLE I**

Sequence and functional data of all functional mutant receptors isolated from a library of 1,200 unique receptors containing randomly introduced mutations in TM6.

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Antagonist atropine. Table I summarizes these data. The receptor tolerated multiple, non-conservative mutations to 14 of the 21 residues in TM6 without severely compromising its ability to be activated by carbachol. These side chains are therefore not essential either for receptor activation by carbachol or for maintaining the overall structure of the receptor. We combined these data with data from an analogous study on the cholerasic receptor (15, 16); however, the effects of mutating residues where mutations were either never seen or were always associated with a greater than 5-fold decrease in carbachol potency. Bold text indicates residues that were never mutated except in receptors showing a greater than 500-fold decrease in carbachol potency. Plain text, Leu-463 was non-conservatively mutated only in receptors showing a greater than 5-fold decrease in carbachol potency. Constitutively activating residues: five residues that were positively identified as sites where point mutations can cause constitutive activity (see Fig. 3, A and B).

In contrast, six residues were identified that either tolerated multiple, non-conservative mutations or were only mutated in severely compromised receptors. Trp-455, Tyr-458, and Val-464 were only mutated in receptors where the potency of carbachol was decreased by over 500-fold, and mutations to Thr-454, Pro-457, and Val-462 were never seen in any of the functional mutants. As the library of 1,200 receptors certainly contained a wide range of receptors that were mutated at these positions, it follows that the receptors which were mutated at these residues were inactive, whether through decreased expression, misfolding or inability to bind or respond to carbachol. The exact side chains of these residues are therefore likely to be important for normal muscarinic receptor expression or function. For Trp-455, Tyr-458, Thr-454, and Pro-457, these results are consistent with earlier data from site-directed mutagenesis of the closely related m3 muscarinic receptor (15, 16); however, the effects of mutating Val-462 and Val-464 have not been previously examined. As shown in Fig. 1C, these functionally conserved residues cluster on one face of TM6. We suggest that this region is involved in internal protein-protein and protein-ligand interactions neces-
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Constitutive activity and maximum response are expressed as a percentage of the maximum response of the wild-type receptor to carbachol. Values represent the mean of at least two determinations and are rounded to 10% or one significant figure.

| Clone | Constitutive activity | Maximum response | EC$_{50}$ Carbachol | EC$_{50}$ Atropine | K$_I$ Atropine |
|-------|-----------------------|-------------------|---------------------|-------------------|---------------|
|       | %                     | %                 | $\times 10^{-9} M$  | $\times 10^{-9} M$ | M             |
| F451V | 90                    | 180               | 0.2                 | 0                 | 10            |
| F451L | 60                    | 160               | 0.2                 | 0.8               | 3             |
| F451T | 90                    | 240               | 0.7                 | 4                 | 10            |
| Wild-type | 10               | 100               | 2                   | 4                 |               |
| F451C | 40                    | 150               | 3                   | 20                | 60            |
| F451A | 40                    | 200               | 3                   | 40                | 60            |
| F451Q | 20                    | 180               | 5                   | a                 | 100           |
| F451N | 40                    | 120               | 70                  | a                 | >1000         |
| F451G | 10                    | 120               | 100                 | a                 | >1000         |
| F451W | 0                     | 60                | 100                 | a                 | 1             |
| F451P | b                     | 120$^b$           | 200                 | 6$^b$             |               |
| N459S | 30                    | 150               | 0.6                 | 80                | 100           |
| Wild-type | 9                   | 100               | 1                   | a                 | 1             |
| N459H | 50                    | 110               | 4                   | 400               | 200           |
| N459Y | 7                     | 110               | 7                   | a                 | >1000         |
| N459K | 0                     | 110               | 9                   | a                 | >1000         |
| N459T | 3                     | 130               | 10                  | a                 | 200           |
| N459Q | 3                     | 100               | 10                  | a                 | 5             |
| N459C | 20                    | 180               | 20                  | a                 | 1000          |
| N459D | 10                    | 120               | 20                  | a                 | >1000         |
| N459A | 20                    | 180               | 30                  | a                 | 400           |
| N459G | 8                     | 140               | 50                  | a                 | 300           |
| N459V | 8                     | 100               | 200                 | a                 | 200           |
| N459F | 6                     | 150               | 200                 | a                 | 200           |

* Atropine EC$_{50}$ values could not be accurately measured because of low constitutive activity.
* F451P produced a positive response to atropine equal to 70% of the wild-type receptor’s response to carbachol. The basal activity level of F451P suggests its constitutive activity is low (<20%).

For receptor folding and activation, the associated cluster of functionally conserved residues in C-i3 has been extensively characterized and plays important roles in G-protein activation (9, 25).

Examination of Table I also reveals a pattern in the mutations within TM6 that cause constitutive activity. All six of the most activated receptors had mutations to either Phe-451 or Ser-465, suggesting that mutations at these sites can cause constitutive activity. In addition, two constitutively activated mutants containing single point mutations were identified: A450V and I447S; thus, mutations at these positions can certainly cause constitutive activity. The phenotypes of these two mutants are compared with the wild-type receptor in Fig. 2A. The basal activities of the mutant receptors were substantially higher than that of the wild-type. Atropine had a strong negative effect on the activity of the mutants, but little effect on the wild-type. Carbachol increased the activity of both the wild-type and mutant receptors, but acted on at much lower concentrations (i.e., had higher potency) on the constitutively activated mutants compared with the wild-type. As shown in Table I, an increase in the potency of carbachol was observed in the majority of the constitutively activated mutants. In contrast, the potency of the antagonist atropine was similar for most of the constitutively activated receptors.

A unique phenotype was seen in a receptor with the three mutations F451L, N459H, and S465C. This receptor had one of the highest levels of constitutive activity, but unlike most of the other activated receptors the potency of carbachol was not increased. Strikingly, the potency of atropine was dramatically decreased (4,000-fold). As described above, it is probable that the constitutive activity of this mutant is caused by the mutations to Ser-465 and Phe-451; however, as other receptors having these substitutions did not have decreased potencies for atropine, it is likely that the change in atropine potency is caused by the mutation in Asn-459.

Site-directed Mutagenesis Confirms the Identity of Constitutively Activating Sites and Reveals Roles for Asn-459 and Phe-451 in Ligand Binding—To characterize the functional properties of important residues identified in this study, we made extensive series of point mutations to Ser-465, Phe-451, and Asn-459. As we have reported previously (20), 12 amino acid substitutions to Ser-465 were tested, and most of these were found to cause increases in both constitutive activity and agonist potency. Bulky and basic substitutions caused the largest effects. The observed constitutive activity could be suppressed by atropine, which acted with similar potency on all the receptors. Careful mathematical analysis of these results revealed that the observed changes in carbachol potency could be explained as a consequence of increased constitutive activity, with no suggestion of any direct contact between Ser-465 and carbachol. As described in detail elsewhere (e.g., Ref. 26), agonists activate receptors by preferentially binding to and stabilizing an active receptor conformation, whereas antagonists preferentially bind to and stabilize an inactive conformation.
Constitutive activity occurs when unliganded receptors exist in an active conformation, and is seen at low levels even in wild-type muscarinic receptors (27–30). Mutations that increase constitutive activity increase the proportion of receptors in the active conformation. As this conformation has a higher affinity for agonists, agonists act with higher potency on more strongly constitutively activated receptors.

Like Ser-465, most mutations to Phe-451 caused significant levels of constitutive activity that could be suppressed by atropine (Table II, Fig. 3B); however, there was no correlation between agonist potency and constitutive activity. For example, the mutant F451N was strongly constitutively activated but the potency of carbachol was dramatically decreased. This phenotype suggests that the mutation F451N disrupts the receptor's interactions with agonists in addition to causing constitutive activity. No structure-activity relationship was seen linking amino acid substitutions to Phe-451 to a particular phenotype. An extraordinary result was seen for the mutant F451P; in this case, atropine actually induced an agonist-like response from the receptor. Fig. 2C illustrates the ability of two antagonists, atropine and quinuclidinyl benzilate (QNB), to stimulate an agonist-like response from F451P.

Asn-459 differed from Ser-465 and Phe-451 in that, of the wide range of substitutions tolerated at this residue, only histidine and serine caused significant increases in constitutive activity. With the exception of the very conservative mutation N459Q, all mutations to Asn-459 caused decreases of over 100-fold in atropine potency (Table II, Fig. 2D), indicating that an amide residue is required in this position for high affinity atropine binding. Mutation of Asn-459 also caused large decreases in the potency of QNB, an antagonist that, like atropine, contains both an ester and a hydroxyl moiety (data not shown). In contrast, the potency of benzotropine (an atropine analogue that has neither of these functional groups) was affected less than 10-fold by most of these mutations. These data suggest that Asn-459 interacts with the hydroxyl and/or ester moieties of atropine (as suggested in Ref. 17). Here, we have demonstrated a strict requirement for an amide group at position 459. This, coupled with the drastic decreases in atropine potency observed when this residue is mutated, suggests that in the wild-type receptor, Asn-459 makes two hydrogen bonds with atropine.

Carbachol potency was also decreased by mutation of Asn-459. Even the constitutively activated receptors N459S and N459H failed to show the large increases in agonist potency normally characteristic of activated receptors (20, 26, 28). The smallest effects on carbachol potency were seen when Asn-459 was replaced with other residues capable of donating hydrogen bonds (e.g. histidine, lysine, and serine) compared with non-donating residues (e.g. phenylalanine and valine). We therefore suggest that a hydrogen bond donated by Asn-459 contributes to receptor activation by carbachol. The smaller decreases seen in carbachol potency relative to atropine may reflect that only one bond is affected.

Conceivably, a decrease in carbachol potency could result from many causes, for example the loss of a receptor-ligand interaction; a nonspecific, structural effect; or steric interference with the carbachol binding site. Nonspecific structural effects are unlikely because the majority of the mutant receptors bind benzotropine with normal affinity. Nonspecific structural effects on the active conformation only are also unlikely because most
of the mutant receptors retain similar levels of constitutive activity to the wild-type. In addition, many mutations to Asn-459 actually increase the potency and efficacy of the agonist McN-A-343 (data not shown); hence, the mechanism of receptor activation is also probably unimpaired. We therefore suggest a direct contact between Asn-459 and carbachol. Asn-459 is conserved in all five muscarinic acetylcholine receptor subtypes, yet is replaced by phenylalanine in all catecholamine receptors. Modeling experiments have predicted that Asn-459 is perfectly positioned to donate a single hydrogen bond to the ester moiety yet is replaced by phenylalanine in all catecholamine receptors. Asn-459 actually increase the potency and efficacy of the agonist activity to the wild-type. In addition, many mutations to Asn-459 are also constitutively activating. Together, these results suggest that the relative orientations of TM3 and TM6 may change on receptor activation such that the cytoplasmic tail of TM3 can interact with G-proteins. As ligand interactions with TM3 and TM6 stabilize the receptor in its active or inactive conformations, TM6 is acting as a ligand-dependent switch.

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