Role of Transmembrane Helix IV in G-protein Specificity of the Angiotensin II Type 1 Receptor*

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G-protein activation by G-protein coupled receptors (GPCRs) is accomplished through proper interaction with the cytoplasmic loops rather than through sequence-specific interactions. However, the mechanism by which a specific G-protein is selected by a GPCR is not known. In the current model of GPCR activation, agonist binding modulates helix-helix interactions, which is necessary for fully determining G-protein specificity and stimulation of GDP/GTP exchange. In this study, we report that a single-residue deletion in transmembrane helix IV leads the angiotensin II type 1 (AT₁) receptor chimera CR17 to retain GTP-sensitive high affinity for the agonist angiotensin II but results in complete inactivation of intracellular inositol phosphate production. The agonist dissociation profile of CR17 in the presence of guanosine 5′-3-O-(thio)triphosphate suggests that the activation-induced conformational changes of the chimeric receptor itself remain intact. Insertion of an alanine at position 149 (CR17-149A) in this chimera rescued the inactive phenotype, restoring intracellular inositol phosphate production by the chimera. This finding suggests that in the wild-type AT₁ receptor the orientation of transmembrane helix IV residues following Cys¹⁴⁹ is a key determinant for effectively distinguishing among various structurally similar G-proteins. The results emphasize that the contacts within the membrane-embedded portion of transmembrane helix IV in the AT₁ receptor is important for specific G-protein selection.

The octapeptide hormone angiotensin II (Ang II)¹ (DRVYI-HPF) mediates diverse biological functions through multiple signals (1–4). Two distinct integral membrane receptors, type 1 (AT₁) and type 2 (AT₂), belonging to the G-protein-coupled receptor (GPCR) superfamily, mediate the intracellular response to Ang II. The predicted secondary structures of AT₁ and AT₂ are characterized by seven transmembrane α-helical segments (TM-I through TM-VII), three connecting loops on either side of the membrane, an extracellular N terminus, and a cytoplasmic C terminus. The extracellular loops and the transmembrane domain are involved in binding Ang II, whereas the cytoplasmic loops form the site for binding the G-protein (guanine nucleotide-binding regulatory protein) upon activation. The AT₁ receptor is a prototypical GPCR, mediating most known functions of Ang II, and the mechanism for the functions of the AT₂ receptor are relatively well studied (3–5, 7–10). The AT₁ receptor is believed to transduce cell growth inhibitory signals and may regulate apoptosis during embryonic development and tissue remodeling in the adult (11–15). The molecular basis for the AT₂ receptor function is largely unknown. Notably, the binding of the agonist Ang II to the AT₂ receptor is insensitive to analogues of GTP, and second-messenger assays for detection of AT₂ receptor activation have been unsuccessful in various recombinantly expressed surrogate cell systems (10–14).

Analysis of the primary structures reveals a 32% amino acid sequence homology between AT₁ and AT₂ receptors (11, 12), but several chimeras of AT₁ and AT₂ receptors (constructed to elucidate the molecular basis of the subtype-specific functions of the Ang II receptor) were fully active. However, a chimera in which AT₂ receptor residues 99–359 have been replaced with a topologically identical segment from the AT₁ receptor was inactive, whereas a chimera with residues 165–359 replaced with a segment from the AT₂ receptor was fully active (Fig. 1). Members of the GPCR family are believed to have the same basic molecular architecture. Therefore, one can construct chimeras that retain distinct subtype-specific functions or exhibit functions of both parent subtypes simultaneously or that display unique defects. The study of chimeric receptors helps us understand the signal transduction mechanism. To gain potentially important insight into the mechanism of G-protein recruitment in Ang II signaling, we analyzed several chimeras containing portions of AT₂ receptor segments. The results indicate that the integrity of TM-IV of the AT₁ receptor is critical for specific G-protein coupling.

EXPERIMENTAL PROCEDURES

Materials—The monoclonal antibody 1D4 was supplied by the Cell Culture Center, Endotronic Inc. Oligonucleotides were obtained from the oligonucleotide synthesis core facility of the Lerner Research Institute, The Cleveland Clinic Foundation. Ang II and [Sar¹,Ile⁸]Ang II were obtained from Bachem. [Sar¹,Ile⁸]Ang II was iodinated by the lactoperoxidase method and purified, as described earlier (15). The specific activity of the [¹²⁵I]-[Sar¹,Ile⁸]Ang II was 2200 Ci/mmol. Losartan was a gift from DuPont Merck Pharmaceutical Co. FD123319 was purchased from Research Biochemicals International. Losartan was from Amersham Pharmacia Biotech. Dithiothreitol and other chemicals were from Sigma.

Cloning, Mutagenesis, and Expression of Ang II Receptors—The synthetic rat AT₁ receptor gene, cloned in the shuttle expression vector pM13, was used for expression as described earlier (5, 7–10, 16). The cDNA of the AT₂ receptor was cloned from mRNA isolated from adrenal medulla of spontaneously hypertensive rat by reverse transcriptase-coupled polymerase chain reaction. The cloned cDNA was fully sequenced and modified for expression in COS1 cells (1) contain a consensus Marilyn-Kozak sequence and a unique EcoRI site at the 5′ end and NotI site at the 3′ end of the gene (2) and to encode an octapeptide
concentrations of GTP

time, either cold Ang II or a mixture of cold Ang II and GTP

was added, and the incubation was allowed to continue for another 60

was taken in transfected. COS1 cells (American Type Culture Collection), cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, were transfected by the DEAE-dextran method. Transfected COS1 cells cultured for 72 h were harvested. Cell membranes were prepared by the nitrogen PARR bomb disruption method and suspended in hypotonic buffer (HME) (50 mM HEPES, pH 7.2, 12.5 mM MgCl2, 1.5 mM EGTA) containing 10% glycerol as described earlier (5, 7–10). The receptor expression was assessed in each case by immunoblot analysis (data not included) and by 125I-[Sar1,Ile8]Ang II saturation binding analysis.

Ligand Binding Study—The ligand binding experiments were carried out under equilibrium conditions as described before (5, 7–10). Briefly, membranes expressing receptors were incubated with 0.3 nM 125I-[Sar1,Ile8]Ang II and 0.03 to 3.0 nM 127I-[Sar1,Ile8]Ang II in assay buffer for the competition binding study. For the saturation binding study, a 10-fold higher concentration of 125I-[Sar1,Ile8]Ang II than the Kd value of the receptor was used to get >90% bound form of the receptor. Nonspecific binding (which was <5% of total binding in our experiments) to the membranes was determined from 125I-[Sar1,Ile8]Ang II binding in the presence of 10−5 M cold 127I-[Sar1,Ile8]Ang II. All binding studies were performed at 22°C for 1 h unless specifically indicated. The binding reaction was stopped by filtering under vacuum (Brandel Type M-24R) on FP-200 GF/C filters (Whatman Inc.). Filter-bound 125I-[Sar1,Ile8]Ang II was quantitated in a gamma-counter (Packard). Equilibrium binding kinetics were determined using the computer program Ligand. The Kd values represent the mean ± S.E. of three or more independent determinations.

GTPγS-dependent Affinity Analysis—To measure the competitive dissociation of 125I-Ang II from the receptors in the presence and absence of G-protein activation receptor bound radioligand was challenged with cold Ang II or a mixture of cold Ang II and GTPγS. To ensure that the 125I-Ang II binding reaction reached equilibrium, the radioligand and membrane were mixed for 30 min at 22°C. After this time, either cold Ang II or a mixture of cold Ang II and GTPγS (100 μM) was added, and the incubation was allowed to continue for another 60 min at 22°C. At the end of the incubation period, the membranes were collected by filtration and washed, and the bound radioactivity was determined. In a typical experiment, the total 125I-Ang II per tube was 330,000 cpm, of which specific binding without any competitor and GTPγS added was 28,000 cpm and the nonspecific binding in the presence of 10−4 M Ang II was 960 cpm. To measure the effect of GTPγS on Ang II binding, membrane samples (10–15 μg of protein) were incubated with 300 pM 125I-Ang II in the presence and absence of varying concentrations of GTPγS. Dissociation of the radioligand was initiated by adding 10−6 M cold 125I-Ang II. The dissociation constants were calculated by nonlinear regression using single or double exponential decay equations.

Production of Inositol Phosphate (IP)—Transfected COS1 cells were cultured in 60-mm Petri dishes for 24 h after transfection, then labeled for 24 h with [3H]myoinositol (1.5 μCi/Petri dish), specific activity 22 Ci/mmol (Amersham Pharmacia Biatech), at 37°C in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum. At 48 h after transfection, the labeled cells were washed with serum-free medium three times and incubated with Dulbecco’s modified Eagle’s medium containing 10 mM LiCl for 20 min; agonists were added, and incubation was continued for another 45 min at 37°C. At the end of incubation, the medium was removed, and total soluble IP was extracted from the cells by the perchloric acid extraction method, as described previously (8–11). The amount of [3H]IP eluted from the column was counted, and a concentration response curve generated using iterative nonlinear regression analysis (see Refs. 8–10 and 15 for additional details). The results of IP production were examined by unpaired Student’s t test, and p values less than 0.05 were considered significant.

RESULTS

Ang II Receptors in COS1 Cell Model System—Recombinant expression in transiently transfected COS1 cells was employed for analysis of the structure-function relationship of the wild-type and chimeric AT1 or AT2 receptors as described earlier (5, 7–10). Expression, in each case, was measured by immunoblotting with the C-terminal epiderm-directed monoclonal antibody 1D4 (data not shown), followed by 125I-[Sar1,Ile8]Ang II binding and competition binding to subtype-specific antagonists (Table I) and then by second-messenger production to measure function. The affinity of the expressed AT1 and AT2 receptors were 0.3 ± 0.09 and 0.21 ± 0.05 nM, respectively, for the nonselective peptide antagonist 125I-[Sar1,Ile8]Ang II and 11.8 ± 1.7 and 17.2 ± 6.4 nM, respectively, for losartan and PD123319. The Kd values of the AT1 receptor for the agonists [Sar1]Ang II and CR17 were 0.33 ± 0.05 nM, respectively, for losartan and 0.16 ± 0.05 nM, respectively, for PD123319. The Kd values estimated for the two receptors were very similar (~5 pmol/mg). Scatchard plot analysis indicated a single affinity class for both receptors. Competition binding studies employing Ang II, [Sar1]Ang II, losartan, and PD123319 demonstrated that the receptors expressed in COS1 cells preserve the selectivity and affinity profiles previously described for native tissue receptors as well as recombinantly expressed receptors. The protein expression of all the mutants described in this report was within 25% of the level of the expression of the wild-type receptor (Table I). The variation in cell surface receptor number, estimated by acid labile binding of 125I-[Sar1,Ile8]Ang II in intact cells, was 1.3–1.7 × 105 sites per cell in this study.

The ability of AT1 receptor to activate IP production in COS1 cells has been described before by our laboratory (5, 7–10). The basal IP production in transfected COS1 cells without [Sar1]Ang II treatment is 5 ± 0.5% (~4000 cpm) when compared with the maximal IP response elicited by [Sar1]Ang II concentration >10−7 M (taken as 100%). This value is significantly higher than that measured in the mock-transfected cells (Fig. 2). Alterations in the functional activity of different mutant receptors could be accurately measured in the COS1 cells, because the maximal IP response elicited by the AT1 receptor is

| Receptor | Kd (nM) | Ang II | PD123319 | Bmax (pmol/mg protein) |
|----------|---------|--------|----------|-----------------------|
| AT1-WT | 0.3 ± 0.09 | 11.8 ± 1.7 | 3.1 ± 0.33 | >10−7 | 5.4 ± 0.12 |
| AT2-WT | 0.21 ± 0.05 | >10−6 | 1.2 ± 0.12 | 17.2 ± 6.4 | 5.2 ± 0.14 |
| CR5 | 3.81 ± 1.30 | >10−6 | ND | 112 ± 28 | 5.0 ± 0.13 |
| CR7 | 6.32 ± 1.84 | >10−6 | ND | 2 ± 10−4 | 4.3 ± 0.16 |
| CR9 | 3.51 ± 1.22 | 6.72 ± 10−6 | ND | 4.1 ± 0.11 |
| CR17 | 2.32 ± 0.64 | 45.2 ± 4.6 | 4.9 ± 0.51 | >10−7 | 4.9 ± 0.28 |
| CR5V1494A | 3.78 ± 1.20 | >10−6 | ND | 120 ± 27 | 4.7 ± 0.21 |
| CR7V1494A | 6.40 ± 1.81 | >10−6 | ND | >10−4 | 5.1 ± 0.19 |
| CR17V1494A | 2.14 ± 0.61 | 42.3 ± 5.4 | 3.4 ± 0.41 | >10−7 | 4.2 ± 0.23 |
AT₁ Receptor G-protein Specificity

**Fig. 1.** A, a secondary structure model of the rat AT₁ receptor. Residues that are conserved in the AT₁ receptor are shaded. Residues 99 and 185, which mark the border of the segment analyzed in the current study, are indicated. Residues 146–165 in the TM-IV, which are replaced with the corresponding region (162–180) of the rat AT₂ receptor, are boxed. Residue Cys146, which is deleted in the resulting chimera receptor (CR17), is indicated by an asterisk. Dashed lines indicate two disulfide bonds on the extracellular side. B, schematic illustration of the putative secondary structure models for the wild-type and chimeric Ang II receptors. The residue number(s) indicated in each case represents the AT₁ receptor position at which the chimeras are joined.

The AT₁/AT₂ Receptor Chimera CR17 Is Inactive in IP Production—We constructed 25 chimeras of AT₁ and AT₂ receptors to elucidate the molecular basis of the Ang II receptor subtype-specific functions. A chimera containing the AT₁ receptor in which residues 99–359 had been replaced with a topologically identical segment from the AT₂ receptor was inactive, whereas a chimera with residues 165 through 359 replaced with that of AT₂ receptor was fully active. Both of these chimeras express well and bind Ang II with high affinity.²

Analysis of chimeras CR5, CR7, CR8, and CR17 (Fig. 1) suggests that the dominant loss of function is linked to the TM-IV segment derived from the AT₂ receptor. These chimeras were constructed to individually evaluate the role of TM-III, TM-IV, and the interhelical cytoplasmic loop connecting the two helices that include residues 99–165 of the AT₁ receptor. The expression levels of the chimeras were comparable to that of the wild-type AT₁ receptor (Table I). The affinity of [Sar¹Ile⁸]Ang II was slightly reduced, and the affinity of losartan was reduced more than 100,000-fold in all of the chimeras. The affinity of PD123319 varied between chimeras.

Chimeras CR5 and CR7 were inactive (Fig. 2), suggesting that the second cytoplasmic segment (CR5) nor the TM-III and the second cytoplasmic segment (CR7) from the AT₁ receptor could rescue the function. The chimera CR8, which contains TM-III from the AT₂ receptor but the second cytoplasmic loop and TM-IV segment of the AT₁ receptor, demonstrated increased (12%) basal activity and was fully activated by [Sar¹]Ang II. The putative mechanism of constitutive activity of CR8 has been fully described previously (9). Restoration of function in CR8 (which contains TM-IV and the adjacent loop segment of the AT₁ receptor) implies that the defect in CR5 and CR7 function is related to the TM-IV segment derived from the AT₂ receptor.

This conclusion is further supported by results from the CR17 chimera, in which the TM-IV region in the AT₁ receptor (Lys¹⁴⁶–Ile¹⁶⁶ shown within the box in Fig. 1A) was replaced by a topologically identical 19-residue segment (Tyr¹⁶²–Tyr¹⁸⁰) of the AT₂ receptor. Although chimera CR17 expressed well and its ligand affinity profile is comparable to that of the wild-type receptor (Table I), it did not activate IP production upon Ang II stimulation (Fig. 2). Because a larger segment exchange (i.e. residues 166–359) between the two receptors yielded functional chimeras, the lack of function in CR17 indicates incompatibility of TM-IV helices in the two Ang II receptors.

The inability of CR17 to stimulate IP production could be caused by the lack of Ang II-induced isomerization to the active state or by the inability of the activated form of the receptor to interact with G-proteins. To distinguish between these two possibilities, G-protein interaction with receptor was evaluated by measuring the dissociation rate of [¹²⁵I]Ang II from wild-type and CR17 chimeric receptors in the presence and absence of added GTPγS (Fig. 3A). In the absence of added GTPγS, the higher affinity of the [¹²⁵I]Ang II in the ternary agonist-receptor-G-protein complex is indicated from 6–10-fold higher molar concentration of the cold Ang II at which the radioligand binding is inhibited. The magnitude of the GTP-induced affinity shift is more pronounced with the CR17 chimera than with the wild-type receptor, perhaps suggesting that different G-proteins are responsible for the shift observed in each. This suggestion needs further confirmation. In the presence of the added GTPγS, the dissociation of [¹²⁵I]Ang II is faster from both wild-type and CR17 chimeric recep-
tors, suggesting the existence of a low affinity binary agonist-receptor complex. This implies that CR17 chimera is capable of stimulating GDP/GTP exchange in G-protein bound to the receptor. The GTPγS-dependent destabilization of the ternary complex implies that the binding and dissociation of G-protein from the agonist-activated receptor is intact. Fig. 3B shows that the dissociation kinetics exhibited by the CR17 chimera is comparable to that exhibited by the wild-type receptor, suggesting that the CR17 receptor interacted productively with a G-protein, even though the IP formation was completely defective. These observations suggest that the ligand activated CR17 chimeric receptor couples to a G-protein from a family other than the phospholipase C (PLC) activating Gq family of G-proteins. Pertussis toxin treatment did not unmask PLC activation in CR17-transfected cells following [Sar1]Ang II stimulation. Thus, the identity of the G-protein coupled to CR17 and of the downstream signal activated is currently unknown.

Because the CR17 chimera contains all the intracellular loops of the AT1 receptor presumed essential for G-protein interaction, it is able to couple to a G-protein other than Gq. We speculate that this defect must be the result of disruption of the G-protein selection mechanism in the CR17 chimera. The homology between the two TM-IV segments of the AT1 and AT2 receptors is 40% (8 out of 20 residues conserved), which is greater than the overall homology (32%) between the two receptors (Fig. 4A). Among the eight conserved residues, Trp153, Ala156, and Pro162 are present in 90% of the members of the GPCR superfamily (see Fig. 4A for conservation of residues). 3

Anchoring these residues, sequence alignment reveals, perhaps most strikingly, a single-residue deletion at position Cys149 upon segment replacement (Fig. 4A). Rather interestingly, this is the only deletion within all seven TM domains when the primary sequence of two receptors is aligned. The remaining

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3 S. Chang and S. S. Karnik, unpublished observations.

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**Fig. 3.** Effect of the nonhydrolyzable GTP analogue GTPγS (100 μM) on 125I-Ang II binding to membrane preparations expressing wild-type and chimeric Ang II receptors (see under “Experimental Procedures” for details). Each data point represents the mean ± S.D. obtained from three or more separate experiments at 22°C. A, competition displacement with and without GTPγS. B, dissociation in the presence of GTPγS. The ratio of high affinity to low affinity receptors estimated from the dissociation curve in the presence of 0.01 μM GTPγS was 3.5:1 in the case of the wild-type, 9:1 in the case of the CR17, and 4.8:1 in the case of CR17→149A. The ratio at 100 μM GTPγS was 0.5:1 in the case of wild-type, 1.1:1 in the case of CR17, and 0.6:1 in the case of CR17→149A.
changes resulting from replacement include substantial differences in residue size due to the Ile→Pro, Leu→Cys, Gly→Cys, Ala→Ser, and Ala→Thr alterations; in charge due to the Lys→Tyr alteration; and in aromaticity due to the Tyr→Val, Val→Phe, and Ile→Tyr alterations. These differences are expected to perturb helical packing. Because the peptide agonist specificity of CR17 is not substantially different from that of the wild-type AT1 receptor, these residue changes are unlikely to be responsible for inducing the functional defect through perturbation in the ligand-exposed phase (Fig. 4B). Finally, it is clear that the changes observed in CR17 do not represent a global unfolding of the protein compared with the wild-type.

**Insertion of an Ala at Position 149 of CR17 Fully Rescued the Function**—To examine whether the predicted deletion of a single amino acid residue was responsible for the inactivation of CR17 in IP production, the CR17→149A mutant was prepared by insertion of an Ala at position 149 of CR17. The CR17→149A mutant receptor activated IP production as much as did the wild-type AT1 receptor (Fig. 2). The ligand affinity profiles for the mutant were identical to that of CR17 and were distinguishable from the wild-type AT1 receptor. As shown in Fig. 3B, the GTPγS-induced dissociation of [125I]Ang II from the CR17→149A chimera closely resembled the profile of wild-type AT1 receptors. Insertion of an Ala at the identical position in the CR5 (CR5→149A) and CR7 (CR7→149A) chimeras fully rescued Ang II-dependent IP production (Fig. 2). In each instance, the ligand affinity profiles resembled that of the inactive chimera (Table I). Strikingly, the CR5→149A chimeric receptor exhibited partial constitutive IP production, comparable to that exhibited in the CR8 chimera, consistent with our previous findings (9).

Thus, the defective phenotype resulting from the single-residue deletion in TM-IV is dominant over the constitutive activation induced by substitution of the TM-III of the AT1 receptor with that from the AT2 receptor. Rescue of function in CR5, CR7, and CR17 with Ala insertion indicates that these chimeric receptors all share the same molecular defect. Furthermore, the results demonstrate that Cys→Ala does not play a specific role in G-protein selection because an Ala substitution also leads to restoration of function. Moreover, Cys is not a highly conserved residue at this position.

**DISCUSSION**

Activation of the AT1 receptor, as in other GPCRs, must involve conformational changes that result from agonist binding within the TM domain of the receptor. The nature of the conformational changes associated with AT1 receptor activation is not known at the present time, but several lines of evidence suggest the occurrence of rather well characterized events similar to those in prototypical GPCRs such as visual rhodopsin and β-adrenergic receptor. For instance, the high affinity state for agonists is believed to result from the formation of a ternary ligand-receptor/G-protein complex, which is
abolished by guanine nucleotides that promote G-protein activation and dissociation of G-protein subunits from the receptor-ligand binary complex. Uncoupling of the receptor from G-protein prevents development of both the ternary complex and the GTPyS-sensitive high affinity state (1–4, 21).

In this study, the functionally inactive AT\textsubscript{1}/AT\textsubscript{2} receptor chimeras formed the ternary receptor-ligand/G-protein complex that demonstrated GTPyS-sensitive dissociation, suggesting that defective IP formation in these chimeras is not because of their inability to adopt an activated conformation or to trigger G-protein activation. Instead, the ability to selectively couple to the G\textsubscript{i}/G\textsubscript{o} pathway upon Ang II-activation is lost. A different G-protein that coupled to the chimeras selectively dissociated upon GTPyS treatment. This observation per se is not novel because several mutagenesis studies of GPCRs have provided examples of relaxed specificity—simultaneous coupling to different G-proteins—by chimeric and mutant receptors, whereas the wild-type receptors exhibit considerable specificity (1–4, 16–19, 21). However, the G-proteins coupled to the AT\textsubscript{1}/AT\textsubscript{2} receptor chimeras were not the pertussis toxin-inactivated G\textsubscript{i}/G\textsubscript{o} proteins, which by their abundance potentially could couple through mass action and block coupling to the G\textsubscript{i}/G\textsubscript{o}-PLC pathway. Furthermore, all three defective chimeras harbored the same defect, which could be overcome by the insertion of a single amino acid in the transmembrane region of TM-IV outside the putative G-protein contact region in all three defective chimeras. These novel and unique characteristics imply that TM-IV of the AT\textsubscript{1} receptor has a role in controlling G-protein specificity.

The third cytoplasmic loop and the C-terminal tail of the AT\textsubscript{1} receptor have been shown to be essential for coupling to the G\textsubscript{i}/G\textsubscript{o}-PLC pathway (3, 22, 23). But the phenotype of CR5\textsubscript{149A}, a chimera that binds the AT\textsubscript{2} receptor-selective antagonist PD123319 and activates AT\textsubscript{1} receptor-specific function, indicates that G-protein specificity is not controlled by these two cytoplasmic regions of the receptor. Instead, the observation suggests that the third cytoplasmic loop and the C-terminal tail of the AT\textsubscript{2} receptor could also support G\textsubscript{i}/G\textsubscript{o}-PLC coupling quite efficiently. All chimeras activated by insertion of Ala\textsubscript{149} contain the second loop and the cytoplasmic extension of TM-IV of the AT\textsubscript{1} receptor beyond the predicted site of deletion, indicating that this region is crucial for G-protein selection. The G-protein-binding specificity of GPCRs is not controlled by discrete consensus sequences in the cytoplasmic loop. Rather, concerted interaction of multiple cytoplasmic segments is required, within a specific and stringent context, for a GPCR to effectively distinguish between various structurally similar G-proteins and productively couple to a specific one. Taken together, the results presented here indicate that the cytoplasmic region of TM-IV could be responsible for controlling G-protein specificity of the wild-type AT\textsubscript{1} receptor. This is likely a general mechanism in the GPCR family in view of the characteristics of TM-IV discussed below.

How did the G\textsubscript{i}/G\textsubscript{o}-PLC uncoupling occur when the particular TM-IV segment (residues 146–166) of the AT\textsubscript{1} receptor was replaced by the topologically equivalent TM-IV segment (residues 162–180) of the AT\textsubscript{2} receptor? The mechanism is not clear in light of the plasticity displayed by TM segments in GPCRs that allow their exchange, sometimes even between distant members, without significantly compromising the function. The mechanism suggested below is based on recent observations in bovine rhodopsin that suggest that rigid body movements of individual TM \(\alpha\)-helices upon light activation (or agonist binding) result in distal changes in structures at the cytoplasmic surface (24, 25). Altenbach et al. (24) determined the topography of the intracellular loop-2 region for bovine rhodopsin. The membrane/aqueous boundary is 3 residues from the conserved Arg in TM-III and 10 residues from the conserved Trp in TM-IV. Baldwin (20) has proposed a model for packing of \(\alpha\)-helices in rhodopsin, which is consistent with the 9 Å electron diffraction density map (6) and the site-directed electron paramagnetic resonance mapping studies. This model is based on helical packing, taking into account the location of highly conserved residues and residue polarity in each of the TM-helices leading to identification of putative surfaces of tertiary interactions. Extending Baldwin’s modeling approach to the AT\textsubscript{1} receptor,\textsuperscript{2} the assumption that length and packing arrangements of helices are the same as in rhodopsin allows us to predict that the TM-IV helical region has a cytoplasmic extension of nearly six residues involved in tertiary interaction with neighboring helices (Fig. 4B). The model predicts that Lys\textsuperscript{146} faces TM-V and perhaps is directly involved in tertiary interaction with TM-V. Electron paramagnetic resonance analysis in rhodopsin indicates that the movement in the TM-IV region in the activated state is limited to <5 Å (there is no vertical displacement with respect to other helices) and that the scope for disengagement of the cytoplasmic extension from the G-protein-interacting surface or reorganization of secondary structure is restricted. This is because TM-IV is unique in that it has no polar-accommodating site in the middle of the hydrophobic core; it has a large lipid-exposed surface with no polar residues that must limit its plasticity. Perhaps due to the lack of flexibility and accommodation of structure, all residues subsequent to the site of deletion (Cys\textsuperscript{149}) in TM-IV would be back-rotated by 100 degrees (Fig. 4B). This anomaly will result in the presence of a new secondary arrangement of residues in the putative G-protein-exposed TM-IV helix/intracellular loop 2 region, which could explain coupling to a different G-protein in the chimeras. This unique defect, which is overcome by a single-residue insertion, implies that the secondary arrangement of residue in the cytoplasmic extension of TM-IV provides a unique interaction surface for G-proteins, which must be preserved for the purpose of specific G-protein selection. The propagation of structural changes toward the ligand pocket and extracellular region may be far less because the agonist affinity profile is only minimally affected, although perturbation in antagonist specificity clearly suggests at least some structural change (Table I). Thus, the rigidity of TM-IV appears to be a key regulatory factor in receptor/G-protein selection.

In summary, substitution in the TM-IV helical segment of the AT\textsubscript{1} receptor with a topologically equivalent segment from the AT\textsubscript{2} receptor results in defective G\textsubscript{i}/G\textsubscript{o}-PLC coupling. The defect is consistent with deletion of a single residue, Cys\textsuperscript{149}, in the TM-IV of the AT\textsubscript{1} receptor. The functional defect caused by deletion of a single residue in TM-IV does not affect agonist interaction or receptor activation but does affect specificity of coupled G-protein. The phenotypes of defective and the cognate reverent chimeras obtained through predetermined insertion of a single residue can be rationalized in terms of displacement of specific residues from a helical structure. The observations reported here suggest that helical movements are remarkably conserved among subfamilies of GPCRs.

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**AT1 Receptor G-protein Specificity**