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MUTAGENESIS OF THE β_2-ADRENERGIC RECEPTOR: HOW STRUCTURE ELUCIDATES FUNCTION

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INTRODUCTION

Accurate responses to extracellular stimuli are one of the most important physiological reactions in every living cell. To accomplish that goal, each cell has developed on its surface numerous highly specialized receptors responsible for transmitting signals from outside the plasma membrane into the cytoplasm.

One of the most thoroughly studied examples of such molecules is the β_2-adrenergic receptor (β_2AR). The β_2AR belongs to the large family of hormone and neurotransmitter receptors that, after binding small biogenic amines (epinephrine or norepinephrine), interact with guanine nucleotide-binding regulatory proteins (G proteins). Activation of the specific G protein G_s causes stimulation of adenylyl cyclase and increases the level of cAMP inside the cell. The signal transduction pathway initiated by the β_2AR and mediated by G_s represents one of the important mechanisms for transmembrane signalling in cellular systems (1).

The cloning of β_2AR cDNA from hamster (2), human (3), rat (4, 5), and mouse (6), as well as the gene from human genomic DNA (7, 8), has allowed

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deduction of the amino acid sequence and analysis of the primary structure of these proteins. The amino acid sequence and proposed membrane topography for the human β_2AR is presented in Figure 1. Hydropathicity analysis suggests that the β_2AR, as well as other membrane-bound, G-protein-linked receptors, are composed of seven hydrophobic regions of 20–25 amino acids each. Based on structural similarities with the better-characterized proteins bacteriorhodopsin and rhodopsin, these hydrophobic domains are proposed to be α-helical in nature and to span the cell membrane seven times. Protease digestion (9) and detailed immunological mapping studies of the β_2AR (10, 11) have provided experimental data that support the predictive value of hydropathicity analyses for this class of membrane-bound receptors with multiple transmembrane-spanning domains. In the present model of G-protein-linked receptor topography, the glycosylated N-terminal domain and the three hydrophilic peptides connecting transmembrane domains II–III, IV–V, and VI–VII are proposed to face the extracellular space, whereas the hydrophilic peptides connecting transmembrane domains I–II, III–IV, and V–VI, as well as the cytoplasmic tail of the receptor are predicted to lie inside the cell. The results of extensive research performed in recent years suggests that the seven transmembrane-spanning domains themselves are involved in ligand binding, whereas the cytoplasmic regions of the receptor are proposed to interact with the G protein G_s.

In this article, we summarize the present knowledge about the structures and functions of the extracellular, transmembrane, and intracellular domains of the β_2AR involved in ligand binding and G-protein coupling. Receptor regions involved in regulatory processes such as desensitization, sequestration, and downregulation have been covered elsewhere (12, 13).

EXTRACELLULAR DOMAINS OF THE β_2AR

N-Terminal Domain—Localization and Possible Role for the N-linked Glycosylation Sites

Results from experiments in which purified β_2AR was treated with different proteases support the hypothesis that the N-terminus of the receptor faces the extracellular side of the plasma membrane (9). In an effort to investigate the role of the N-terminal domain of the β_2AR, Dixon et al showed that deletion of 10 amino acid residues at position 21–30 did not substantially influence ligand binding (14).

Analysis of the N-terminal amino acid sequence of the hamster β_2AR has revealed two consensus sites for N-linked glycosylation (i.e. *N*-X-S/T) at positions 6 and 15, supporting previous findings that the sites of glycosylation are located in this region of the receptor (9). Earlier it had been shown that similarly located sites were glycosylated in rhodopsin (15).

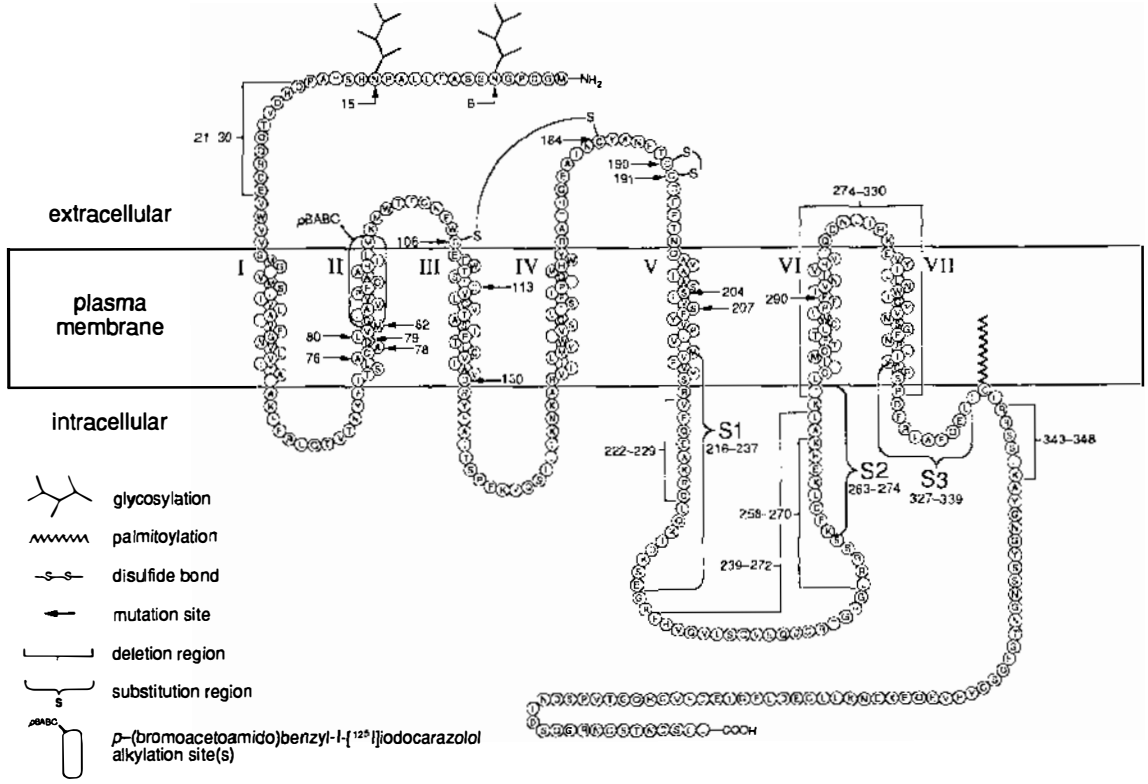

Figure 1 Amino acid sequence and putative membrane topography of the β_2-adrenergic receptor. Important regions and residues are indicated, as described in the text.

Mammalian β_2ARs have been shown to be single-chain glycoproteins of $M_r \sim 65$ kD, with N-linked carbohydrates accounting for approximately one fourth of the apparent molecular weight of the protein (16, 17). Benovic et al showed that treatment of the purified β_2AR with endoglycosidase F resulted in a two-step reduction in the apparent size ($M_r \sim 57$ kD and 49 kD polypeptides). These data are consistent with the notion that two N-linked carbohydrate chains reside on each receptor. Further analysis by exoglycosidase treatment showed the presence of two complex carbohydrate chains on approximately 45% of the receptors. The residual 55% appeared to contain a mixture of carbohydrate chains, possibly high mannose hybrid and complex chains (17). Recently, Rands et al (18) identified three forms of β_2AR-related polypeptides ($M_r \sim 67$ kD, 56 kD, and 43 kD) by immunoblot analysis of cells transfected with the β_2AR cDNA. Enzymatic treatment of membranes from transfected cells demonstrated that the 67 kD polypeptide was sensitive to endoglycosidase F and produced a 43 kD band only. In contrast, the 56 kD band was more sensitive to digestion by endoglycosidase H than to endoglycosidase F, thus strongly suggesting that this polypeptide is a mannose-rich intermediate in the receptor-processing pathway (18).

For many cell surface glycoproteins, glycosylation has been implied to be crucial for the appropriate function and cellular distribution of the protein (19, 20). Neither the treatment of purified β_2AR with endoglycosidase F, nor the growth of receptor-expressing cells in the presence of N-linked glycosylation inhibitors, showed any effect on ligand binding by the β_2AR (17, 21, 22). With site-directed mutagenesis, β_2AR mutants were constructed in which one or both asparagine residues (Asn^6 and Asn^{15}) were either substituted with glutamine or a whole region including residues 6 and 15 was deleted. When transiently transfected in COS cells, these mutants exhibited normal ligand-binding behavior. These findings indicate that the carbohydrate-bearing portion of the receptor does not contribute to the determination of agonist or antagonist binding characteristics. The same mutants expressed in mouse L cells at lower, more physiological levels, however, demonstrated decreased agonist affinity, suggesting perhaps the glycosylation may play a role in receptor-agonist interaction (18).

Similarly controversial is the issue of adenylyl cyclase stimulation mediated by nonglycosylated receptors. George et al reported no effect on the coupling of the β_2AR to G_s and subsequent stimulation of adenylyl cyclase in cells treated with the glycosylation inhibitor tunicamycin (22). Benovic et al also found no change in β_2AR coupling to purified G_s in reconstitution experiments with purified, deglycosylated receptors (17). In contrast, Boege et al reported that receptors synthesized in the presence of tunicamycin showed a reduction in their efficiency of mediating adenylyl cyclase activation (23). Recently, Rands et al (18) showed that β_2AR glycosylation mutants

exhibited a range of impairment in their ability to couple to G proteins and to mediate stimulation of adenylyl cyclase in permanently transfected L cells, as well as a lower affinity for agonist. The authors speculated that this decreased efficiency in coupling might reflect a fundamental requirement for the presence of the carbohydrate moiety in the receptor–G protein interaction or, alternatively, could be due to altered compartmentalization and distribution of the nonglycosylated β_2AR mutants between the cell surface and intracellular vesicles (18).

A series of experiments with hydrophilic and hydrophobic ligands showed that the absence of glycosylation in the β_2AR led to altered intracellular localization of the receptor. Therefore, the current data suggest that the presence of carbohydrate chains on the N-terminus of the β_2AR can be important for proper transport of the receptor to the cell membrane, but it has no obvious effect on ligand binding, coupling to G_s, or subsequent adenylyl cyclase stimulation (17, 18, 22). These findings for the role of N-linked glycosylation of the β_2AR are similar in some respects to those for the insulin receptor, the epidermal growth factor receptor, and the nicotinic actylcholine receptors, where glycosylation has been also shown not to affect the ligand-binding characteristics of the receptors (24).

Hydrophilic Extracellular Domains—Regions Involved in Proper Receptor Folding

Immunofluorescence analysis of the β_2AR has indicated that the three hydrophilic domains predicted to form loops connecting transmembrane-spanning regions II–III, IV–V, and VI–VII face the extracellular space (10, 11; Figure 1). As deduced from the cDNA sequence of the β_2AR, the first and third extracellular loops are rather short whereas the second is longer. The first extracellular loop has one cysteine residue (Cys^{106}) and the second extracellular loop has three cysteine residues (Cys^{184}, Cys^{190}, and Cys^{191}). Mutants constructed with deletions of amino acids 179–187 in the second extracellular loop bound agonist and antagonist with reduced affinity, perhaps implying a role of this region either in the direct binding of the ligand or in the proper folding and transmembrane assembly of the receptor (4, 25). Later studies demonstrated that four cysteine residues (Cys^{106}, Cys^{184}, Cys^{190}, and Cys^{191}) located in the extracellular domains of the β_2AR are involved in disulfide-bridging, important for agonist and antagonist interactions with the receptor (25–27). By assessing ligand binding in the presence and absence of the reducing agent dithiothreitol (DTT), Dohlman et al demonstrated the critical involvement of one, or perhaps two, disulfide bridges in ligand binding to the receptor (27).

In a further effort to delineate the importance of these cysteine residues in β_2AR function, several groups used site-directed mutagenesis to show that

$Cys^{106, 184, 190, 191}$ were directly involved in that process (25–27). Dixon et al had postulated that cysteine residues at positions 106 and 184 formed a disulfide bridge between the first and second extracellular loops of the receptor (25). Dohlman et al reported that following substitution of these extracellular cysteines, binding affinities were reduced 14- to 1400-fold for agonist and 4- to 16-fold for antagonist (27). Cys^{190} and Cys^{191} are proposed to form a vicinal disulfide bond. A vicinal disulfide bond has also been postulated to play an important role in the activation of the nicotinic acetylcholine receptor. The rearrangement of disulfide bonds during posttranslational modification may generate the thermodynamically stable conformation of the receptor required for agonist or antagonist binding (24).

HYDROPHOBIC TRANSMEMBRANE DOMAINS—PROPOSED SITES FOR LIGAND BINDING

Genetic and biochemical approaches have led to the conclusion that amino acid residues found within the hydrophobic core of the receptor are important for ligand binding. This was indicated by examination of a mutated β_2AR, in which amino acids 274–330 from transmembrane α-helices VI and VII were deleted (14, 25). Also, several groups have made detailed assessments of α_2/β_2 and β_1/β_2AR chimeras, in which corresponding transmembrane domains were systematically exchanged between receptors, resulting in conversion of ligand-binding specificity. The importance of helices IV, VI, and VII in particular was noted (28–30). Based on these data, as well as electron diffraction data for bacteriorhodopsin (31, 32), the authors proposed a model in which transmembrane helices are oriented to form a ligand-binding pocket (Figure 2).

The endogenous agonists for the β_2AR are the catecholamines epinephrine and norepinephrine, in which a protonated amine group is separated from the catechol ring by a β-hydroxyethyl chain. Antagonists are characterized by increased hydrophobicity of the aromatic ring and by a greater distance between the ring and the amine group. It is thought that binding between the receptor and ligand must include at least three separate sets of interactions: (*a*) an interaction with the amine group, (*b*) hydrogen bonds with the β-hydroxyl and catechol hydroxyl groups, and (*c*) interactions with the aromatic catechol ring. In efforts to delineate the ligand-binding site in the β_2AR, all conserved polar residues within the hydrophobic domains of the receptor have been systematically mutagenized. Most of the amino acid substitutions did not affect the ligand-binding properties of the receptor. Substitution of Asp^{113} in transmembrane domain III with glutamine or asparagine, however, leads to dramatic decreases in the receptor's affinity for both agonists and antagonists. Comparison of amino acid sequences for the other known G-protein-coupled

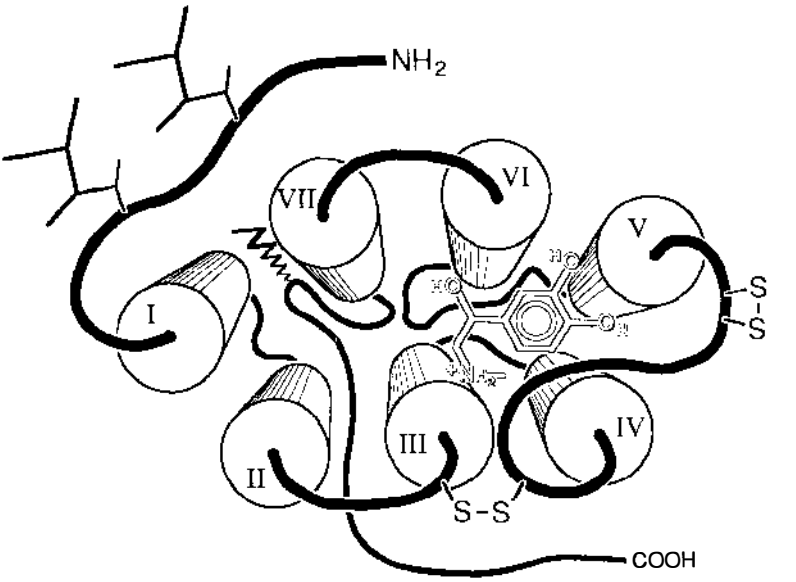

Figure 2 Proposed arrangement for the transmembrane helices of the β_2-adrenergic receptor, depicting the binding site for epinephrine.

receptors that bind biogenic amines reveals that an acidic amino acid is conserved at the equivalent position of the β_2AR Asp 113 in all of them, thus supporting the proposed role for this residue as the counter-ion for the amine moiety of the ligands (33).

In contrast, substitution of the highly conserved Asp^{79} in transmembrane domain II with alanine caused a 10-fold decrease in agonist affinity and adenylyl cyclase stimulation, whereas the affinity of antagonist binding by the mutant receptor was not affected (34). Interestingly, substitution of Asp^{79} with asparagine exhibited normal, low-affinity agonist binding, but was incapable of forming a guanine nucleotide-sensitive, high-affinity binding state for agonist, thus suggesting a functional uncoupling of the receptor from G_s (35, 36). This observation raised the possibility that Asp^{79} is involved in maintaining a receptor conformation necessary for high-affinity agonist binding. Because this residue is predicted to be located in the aqueous core formed by the hydrophilic surfaces of the amphipathic transmembrane helices, it is unlikely that Asp^{79} directly interacts with G_s. Therefore, some other mechanism(s), perhaps agonist-induced conformational changes, must link the influence of this residue to the process of G-protein coupling.

Substitution of the third highly conserved aspartic acid residue (Asp^{130}) within transmembrane domain III with asparagine results in normal antagonist

binding, but significantly higher affinity for agonist than the wild type β_2AR. The effects of guanine nucleotide on the high-affinity agonist binding by this mutant were substantially different from those for the wild type β_2AR, thus suggesting that the coupling of this mutant to G_s was also altered. In addition, this mutant was unable to mediate activation of adenylyl cyclase in response to isoproterenol stimulation (37). These data, together with the results from studies on Asp^{79} mutants, suggest the importance of these residues in direct agonist binding and in agonist-induced conformational changes in the β_2AR that are associated with receptor-G_s interactions.

Detailed genetic and pharmacological analyses have implicated hydrogen-bonding interactions in agonist binding by the β_2AR. The hydroxyl groups of the catecholamine catechol ring have been shown to interact with the sidechains of serine residues at positions 204 and 207 in transmembrane domain V (38). Substitution of either Ser^{204} or Ser^{207} with alanine diminishes agonist-binding affinity approximately 25 to 35-fold, without affecting antagonist binding. Moreover, the effects of serine substitutions on agonist-binding affinity could be selectively emulated in the wild type receptor by the removal of the hydroxyl moieties from the catechol ring of the agonist. The authors concluded that the interaction between the β_2AR and catecholamine agonists requires two hydrogen bonds, one between the hydroxyl group of Ser^{204} and the meta-hydroxyl group of the ligand, and a second between the hydroxyl group of Ser^{207} and the *para*-hydroxyl moiety of the ligand. The fact that both serine residues are highly conserved in G-protein-coupled receptors that bind catechol ligands, but not in those that do not, further supports these conclusions. Similar studies involving the hydroxyl sidechain of Phe^{290} in transmembrane domain VI demonstrated its interaction with the phenyl ring of agonist ligands (39).

To elucidate further the structural determinants of the hormone-binding domain, an interesting line of evidence was presented by Dohlman et al (40). The incorporation of the specific β_2AR alkylating agent *p*-(bromoacetoamido)benzyl-*l*[^{125}I] iodocarazolol (^{125}*l*-pBABC) into the receptor's binding site, showed that amino acid residues 83–96 in transmembrane domain II contain the site(s) of ^{125}*l*-*p*BABC incorporation (40). These data support the hypothesis that the binding site for the hormone lies within a "nest" formed by the hydrophobic transmembrane domains.

Random mutagenesis of the β_2AR, expressed in *Escherichia coli*, brought another novel line of evidence supporting this conclusion (41). Transmembrane domain II, one of the most conserved regions in G-protein-coupled receptors, was targeted for the oligonucleotide-directed random mutagenesis. Random mutations were introduced between amino acid residue 76 and 83 of the β_2AR to examine the pattern of residue substitutions accommodated at given positions while still permitting function. Only conservative substitutions of residues Ala^{76}, Ala^{78}, Leu^{80}, and Met^{82} were found to preserve

ligand-binding characteristics. These residues, therefore, were postulated to be important either in direct binding of the radiolabelled ligand or in supporting the folded structure of the receptor. Interestingly, aspartic acid Asp^{79}, a residue highly conserved between different receptors, was found to be replaced with a variety of amino acids without impairment in ligand binding, thus suggesting that it is not required either for the direct binding of ligands or for the folding and transmembrane assembly of the receptor. The authors concluded that Asp^{79} must play a role in transferring the signal of bound agonist, but not in the structure of the ligand-binding site itself. Experiments examining the binding domain of the β_2AR with the fluorescent antagonist carazolol have shown that the ligand is bound to the receptor in a rigid hydrophobic environment and that the ligand-binding pocket is buried deep within the core formed by the transmembrane helices (42).

HYDROPHILIC INTRACELLULAR DOMAINS—RECEPTOR REGIONS INVOLVED IN G-PROTEIN COUPLING

In rhodopsin, proteolytic digestion of the third intracellular loop inhibits light-dependent coupling to the G protein transducin (43), and activation of the retinal phosphodiesterase (44). By analogy, the putative intracellular domains of the β_2AR were predicted to interact with the G protein G_s. This view has been confirmed by results from site-directed mutagenesis experiments in which different segments of the third intracellular loop and the C-terminal domain of β_2AR were deleted. A large deletion involving residues 239–272 in the third intracellular loop resulted in complete loss of ability to mediate stimulation of adenyl cyclase (14). These data were partially in conflict with studies done by Rubenstein et al that showed that removal of the central part of the third intracellular loop by limited proteolysis did not affect coupling to G_s (45). Rubenstein et al explained the discrepancy by arguing that the large deletion must impose alterations in the tertiary structure of the receptor that result in loss of coupling to G_s. Proteolysis of the wild type receptor, on the other hand, may leave the properly folded structure relatively unchanged and therefore not affect coupling to G_s.

More detailed analysis of the third intracellular loop by limited deletions showed that a small region of eight amino acids at the N-terminal portion of the loop (residues 222–229) was absolutely required for β_2AR-mediated activation of adenylyl cyclase. In addition, deletion of 12 amino acids (residues 258–270) from the C-terminal portion of the third loop markedly impaired the receptor's ability to mediate stimulation of adenylyl cyclase. Several other small deletions constructed in the middle part of the third intracellular loop or within the cytoplasmic tail (amino acids 343–348), as well as a truncation mutant where 60 residues were removed from the

cytoplasmic tail of the receptor (T-354), failed to impair stimulation of adenylyl cyclase (14, 46). These deletions also did not change the ability of the mutant receptors to bind agonist or antagonist. Mutated receptors that could not mediate stimulation of adenylyl cyclase, however, generally bound agonist with a single affinity, in contrast to the wild-type β_2AR, which typically exhibits both high- and low-affinity states for agonist. The presence of high-affinity agonist binding reflects coupling of the receptor to the G protein. Mutated receptors with deletions at either the N- or C-terminal portions of the third intracellular loop bound agonist with a single affinity that was not affected by the addition of GTP analogues (which normally shift agonist affinity due to G-protein dissociation). This, along with the mutated receptors' inability to mediate adenylyl cyclase stimulation, suggested that these receptors were unable to interact with G_s (14, 46).

In a novel experimental approach, Kobilka et al (28) constructed and expressed a series of chimeric α_2/β_2AR genes. The α_2AR and β_2AR are both activated by epinephrine but differ in their G-protein coupling specificities. The β_2AR couples to G_s, a stimulatory G protein for adenylyl cyclase, whereas the α_2AR couples to G_i, an inhibitory G protein for adenylyl cyclase. By investigating the degree of adenylyl cyclase activation mediated by different chimeric α_2/β_2AR, the authors demonstrated that the region extending from the N-terminus of transmembrane domain V to the C-terminus of VI in the β_2AR was sufficient to cause coupling to G_s by the chimeric α_2/β_2AR (28).

Limited primary amino acid sequence similarities between corresponding cytoplasmic domains of the β_2AR and other G-protein-coupled receptors imply that these regions might contain important functional elements for G-protein interaction. Alternatively, the β_2AR amino acid residues involved in the specific interaction with G_s are expected to be different from the corresponding sequences in other receptors responsible for interaction with different G proteins. Based on this logic, O'Dowd et al constructed 19 mutant receptors, in which they replaced different segments of the β_2AR cytoplasmic domains with the corresponding sequences from the α_2AR (47). In agreement with other data, the authors found that the C-terminal segment of the third cytoplasmic loop and the N-terminal portion of the cytoplasmic tail were critical in the formation of the G_s binding site. The short extracellular loop between transmembrane domains VI and VII (i.e. ~5 residues) suggests that these two regions lie in close proximity to each other on the cytoplasmic side of the membrane and may form a binding surface for G_s. It is important to note that the corresponding regions of rhodopsin have been shown to play a role in the interaction with the G protein transducin (48).

Substitution of the conserved residue Cys^{341} in the cytoplasmic tail of the β_2AR causes a significant impairment in the ability of the mutant receptor to

mediate agonist stimulation of adenylyl cyclase (49). In rhodopsin, corresponding vicinal cysteine residues have been shown to undergo palmitoylation (50). Similarly, Cys^{341} in the β_2AR was also proved to be covalently linked by a thioester bond to palmitic acid (49). These data suggest that Cys^{341} is critical in supporting a proper configuration of the N-terminus of the cytoplasmic tail, perhaps in its relation to the C-terminal segment of the third intercellular loop, by promoting formation of a fourth intracellular loop (50) (Figure 1).

A β_2AR mutant constructed by substitution of ten amino acids in the analogous N-terminal portion of the third intercellular loop with corresponding residues from the α_2AR exhibited nearly normal agonist stimulation of adenylyl cyclase as well as an increase in agonist binding affinity (47). These data were in apparent conflict with the results of Strader et al, in which a deletion in the same region of hamster β_2AR caused impairment of adenylyl cyclase stimulation by isoproterenol (46). It was proposed that the amino acid sequences in this region of the third intracellular loops of the β_2AR and the α_2AR share a common structural component for a nonspecific G-protein interaction site. These results still indicated, however, that the N-terminus of the β_2AR third intracellular loop is insufficient to provide G_s versus G_i coupling specificity.

To delineate the receptor domains conferring G_s specificity, five mutants were constructed in which segments of 12–22 amino acids in the β_2AR were substituted with corresponding residues from the α_2AR (51) (Figure 1). In the β_2AR, substitutions in the N-terminal portion (S1, positions 216–237) and C-terminal portion (S2, positions 263–274) or the third intracellular loop and in the N-terminus (S3, positions 327–339) of the cytoplasmic tail (Figure 1) were studied in various combinations. Substitution of region S2 into the β_2AR led to significant impairment in the receptor's ability to couple to G_s. A smaller effect was also observed with substitution of region S3. Similar to the previous results of O'Dowd et al (47), the 22-amino-acid S1 substitution failed to affect the ability of the β_2AR to couple to G_s. Upon combination of all three substitutions, however, a mutant receptor was produced that was capable of coupling to G_i, in addition to a residual ability to couple to G_s. Analysis of the mutant receptors by treatment with pertussis toxin (PTx) suggested that the majority of the impairment in G_s coupling in the absence of PTx was due the competing actions with to PTx-sensitive G_i proteins. In contrast, the impairment of coupling observed in substitution mutants S2 and S3 mutations was interpreted as a result of the inability of these receptors to couple to either G_s or G_i.

It is interesting to note the unique aspects of coupling behavior of S1,2,3 in both functional (i.e. receptor-mediated adenylyl cyclase activity) and physical (i.e. high-affinity agonist binding) assays. Under equilibrium binding con-

ditions, mutant S1,2,3 was able to couple to both G_s and G_i, as reflected in the observation of a high-affinity binding site that was partially sensitive to PTx. Even though the S1,2,3 mutant may show a lower affinity for G_i-GDP than for G_s-GDP, the excess of G_i in relation to G_s believed to exist in the cells (52) should favor G_i-related high affinity agonist binding. When assessed by adenylyl cyclase assays, however, receptor-mediated enzyme inhibition by the mutant S1,2,3 was not observed (51). The authors postulated that this apparent lack of adenylyl cyclase inhibition was masked by concurrent adenylyl cyclase stimulation as a result of sufficiently productive coupling to G_s.

With regard to amino acid sequence and length, the third intracellular loop represents the most divergent domain among G-protein-coupled receptors. There are no clear consensus amino acid sequences to suggest specific sites for G-protein interaction. Secondary structure predictions indicate that regions of the N- and C-terminal portions of the third intracellular loop may form amphipathic α-helices that are extensions of transmembrane domains V and VI. These facts along with data showing that mastoparan, a bee venom peptide that activates G proteins, forms an amphiphilic α-helix in solution (53) led to the hypothesis that the amphipathic character of these α-helices is a critical determinant in the interaction of the β_2AR with G_s. Cheung et al demonstrated that peptides corresponding to the N- and C-terminal portions of the third intracellular loop (14–15 amino acids in length) were able to activate the GTP-ase activity of G_s to the same extent as mastoparan in reconstitution experiments, but did not activate either G_i or G_o (54). Unlike mastoparan, which activated all G proteins to the same extent, these peptides were markedly more effective in activating G_s, thus suggesting that in addition to helical properties, specific amino acids from these regions may directly contribute to the determination of the receptor coupling selectivity for G_s over G_i or G_o. Therefore, it might be anticipated that the mechanism of receptor-mediated G_s activation involves interaction with the amphipathic α-helices from the N- and C-terminal portions of the third intracellular loop that are exposed during agonist-induced conformational changes in the receptor.

Interestingly, the N-terminal portion of the third intracellular loop in the α_2AR that interacts with G_i (28) was also proposed to form an amphipathic α-helix consisting of charged residues similar to those in the β_2AR and hydrophobic residues on the opposite face of the helix that differed from the β_2AR (39). Substitution of β_2AR residues by α_2AR in this region has been shown not to alter the specificity of coupling to G_s or G_i (47, 51). These data suggest that the specificity of the receptor for a particular G protein is determined mainly by helical regions of the third intracellular loop, but can also be encoded in different intracellular domains of the receptor.

This conclusion has been supported by the work of several groups, which tried to delineate other important intracellular regions in different G-protein-

coupled receptors. Palm et al showed that synthetic peptides corresponding to the first and second intracellular loops of the avian β_1AR inhibited the hormone-dependent activation of adenylyl cyclase when assayed in the membranes. In contrast, a peptide from the C-terminal portion of the third intracellular loop was shown to increase adenylyl cyclase activity (55).

In rhodopsin, on other hand, it has been shown that synthetic polypeptides from the second, third, and fourth intracellular loops are capable of interacting with the G protein transducin, either alone or in various combinations. Combination of any two peptides that coupled to transducin individually showed a synergistic effect of 15-fold higher transducin activation (56). By analyzing the effects of mutations in the second and third intracellular loops of rhodopsin, Franke et al proposed that binding of transducin is not sufficient for its activation and that activation of bound transducin requires at least two sites, one in each of the two loops (57). Similar results have also been obtained from studies of various chimeric receptors. Wess et al reported that in chimeric m2/m3 muscarinic acetylcholinergic receptors (AChR), the N-terminal portion of the third intracellular loop was a sufficient but not exclusive determinant of coupling specificity (58). In a very detailed study of chimeric m1AChR/β_1AR receptors, Wong et al (59) demonstrated that replacement of the third intracellular loop of the m1AChR with either the entire third intracellular loop of the β_1AR or a dodecapeptide derived from the N-terminal portion of the β_1AR third intracellular loop conferred the ability to interact with G_s without abolishing the interaction with G_q (the principal target for the m1AChR). The authors also demonstrated that replacement of only the second intracellular loop of the m1AChR with the corresponding loop of the β_1AR diminished stimulation of G_q but did not markedly enhance stimulation of G_s. Substitution of the second intracellular loop did potentiate stimulation of G_s when combined with either of the third intracellular loop substitutions, however, suggesting that both loops interact with each other to select which G protein the receptor will stimulate (59).

Regions in the β_2AR important for G_s coupling other than those found in the third intracellular loop and N-terminal portion of the cytoplasmic tail have also been identified. Via site directed mutagenesis, O'Dowd et al showed that the second intracellular loop was essential for normal β_2AR-G_s interactions, and, in agreement with other data, the role of this loop was concluded to be in maintaining the configuration of the G-protein binding site of the receptor (47). Recently, Cotecchia et al (60) constructed a chimeric α_1/β_2AR, in which the third intracellular loop of the β_2AR had been replaced by the corresponding loop of the α_1AR. They observed that, in addition to an ability to stimulate phospholipase C (the primary effector in the α_1AR signaling pathway), the chimera was still capable of adenylyl cyclase activation, thus suggesting the existence of determinants for G_s specificity in regions other than the third intracellular loop (60).

Presented results strongly suggest that the N- and C-terminal portions of the third intracellular loop are the primary regions responsible for specificity in receptor–G protein interactions. On other hand, the third intracellular loop must clearly act in concert with other cytoplasmic domains of the receptor—i.e. the second intracellular loop or the N-terminal portion of the cytoplasmic tail—to obtain full efficiency and specificity in receptor–G protein coupling.

CONCLUSIONS AND FUTURE DIRECTIONS

Recent cloning of the β_2-adrenergic receptor has accelerated the process of understanding the structure and function of one representative of the large family of G-protein-coupled hormone receptors. The amino acid sequence deduced from cDNA clones has allowed prediction of the secondary and tertiary structure of this receptor. Analysis of amino acid similarities between different G-protein-coupled receptors helps to propose important domains and residues that can then be mutagenized to delineate their involvement in important receptor functions such as (*a*) correct receptor folding and assembly in the cell membrane, (*b*) ligand binding, (*c*) physical and functional coupling to G proteins. Biochemical analysis of purified β_2AR and the physiological properties of many mutants constructed by site-directed mutagenesis, in conjunction with results from similar studies on other G-protein-coupled receptors, has helped build a model in which seven hydrophobic α-helices spanning the cell membrane from a binding pocket for the ligand. The results of other studies has helped to point out specific regions and amino acid residues important in receptor-ligand interactions, providing, for the first time, information that will help us to understand the molecular nature of these processes. Detailed characterization of the intracellular hydrophilic domains by classical biochemical approaches and molecular genetic technology have provided us with extraordinary but still incomplete information about regions involved in the interaction between receptor and G protein. Unfortunately, a limiting factor in understanding the molecular nature of ligand-receptor-G-protein interactions is our rudimentary knowledge of transmembrane protein structure. A major obstacle in the solution of this problem is a lack of sufficient quantities of purified receptor, as well as an established methodology for the crystalization of such proteins. The recent rapid evolution of procaryotic and eucaryotic expression technologies may soon make available sufficient quantities of receptor proteins for crystallization and X-ray analysis. Nonetheless, new molecular biological and biochemical approaches provide powerful tools for further study. Based on analysis of chimeric receptors and small peptides derived from regions previously characterized as important in receptor function, more information about the structure and function of these proteins so important in cell physiology should soon be available. This

information about cellular surface receptors will bring new possibilities and approaches for the design of more useful and effective therapeutic agents, as well as broaden our understanding of the basic physiological processes occurring inside the living cell.

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