Identification of Intramolecular Interactions in Adrenergic Receptors*

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Adrenergic receptors are representative of a large family of plasma membrane receptors that interact with G proteins during the process of transmembrane signal transduction. G protein-coupled receptors have a primary structure that is homologous to bacteriorhodopsin and are proposed to have a similar three-dimensional structure; however, it has not yet been possible to examine this hypothesis experimentally. We have used a novel mutagenesis approach to identify intramolecular interactions. Our results indicate that specific amino acids in the seventh hydrophobic segment of α2 and β2 adrenergic receptors lie adjacent to the first hydrophobic segment. These studies provide the first experimental evidence defining spatial relationships that exist in the three-dimensional structure of adrenergic receptors.

The seven hydrophobic segments that characterize most G protein-coupled receptors are believed to be membrane-spanning domains. Mutagenesis and chimeric receptor studies have shown that the hydrophobic segments of adrenergic receptors form the ligand binding pocket (see reviews: Kobilka (1992) and Ostrowski et al. (1992)). Several structural models have been proposed for G protein-coupled receptors (Dahl et al., 1991; Findlay and Eliopoulos, 1990; Hibert et al., 1991; MaloneyHuss and Lybrand, 1992), and most of these models are based on the structure of bacteriorhodopsin, which has been solved to approximately 10 Å resolution (Henderson et al., 1990). However, none of these models have been tested experimentally. Obtaining a high resolution structure of a G protein-coupled receptor will be a formidable task because of the expense involved in producing and purifying large quantities of receptor protein and the inherent difficulties in obtaining high quality crystals of membrane proteins.

We have attempted to gain insight into the three-dimensional structure of adrenergic receptors by identifying intramolecular relationships. Our approach is to identify complementary mutations which restore functional properties to

mutationally inactivated receptors. The effect of mutations on receptor function may result either from a direct modification of a functional domain or from a more general effect on the three-dimensional structure of the receptor. These more general structural alterations are likely to occur when the mutated domain is in close proximity to another domain of the receptor, particularly if the chemical or physical properties of the mutated amino acid are essential for maintaining the three-dimensional architecture of the protein (Dill, 1990).

We have taken advantage of these principles to study the structure of adrenergic receptors and have obtained evidence that specific amino acids in the seventh hydrophobic segments of the α2 and β2 adrenergic receptors lie adjacent to the first hydrophobic segment in the three-dimensional structure of the protein.

EXPERIMENTAL PROCEDURES

Construction and Expression of Mutant and Chimeric Receptors—The details of the methods used for the construction and expression of mutant and chimeric receptors are described elsewhere (Suryanarayana et al., 1991; Suryanarayana and Kobilka, 1991). The mutant and chimeric receptors were constructed by polymerase chain reaction techniques using human α2C10 and β2 adrenergic receptors cloned into pGEM-3Z vector (Promega Biotec) as templates. The α2 and β2 receptor composition of the chimeric receptors is as follows: CRS11N312 → F (α2(1-106)/β2(107-413))N312 → F; CRS16N312 → F (β2(1-61)/α2(62-106)/β2(107-413))N312 → F; CRS13 (α2(1-63)/β2(64-413))N312 → F; CRS16 (β2(1-61)/α2(62-106)/β2(107-413)); CRS26N312 → N (α2(1-106)/β2(107-264)/α2(333-450))N312 → N; CRS6N312 → N (β2(1-61)/α2(62-450))N312 → N. Wild-type, mutant, and chimeric receptor constructs were transferred from pGEM-3Z to eukaryotic expression vectors pBC12MI and pWS290, and transfected into COS-7 cells and Raji cells by a DEAE-dextran method and electroporation, respectively. Ligand Binding and Adenylylcyclase Assays—The ligand binding and agonist-stimulated adenylylcyclase assays on membranes prepared from Raji cells 9–15 days after transfection were performed as described (Suryanarayana and Kobilka, 1991). Binding assays on transfected COS-7 cells were performed 3 days after transfection.

Immunofluorescence Staining of Transfected COS-7 Cells—The receptor protein in transfected COS cells grow on glass coverslips was localized by indirect immunofluorescence (Suryanarayana et al., 1991; von Zastrow and Kobilka, 1992). Primary antibody was a rabbit antiserum directed to the carboxy-terminal 15 residues of the human β2 receptor, and the secondary antibody was a Texas Red conjugate of goat anti-rabbit IgG. The α2 and α2N312 → F were tagged with an epitope of 9 amino acids at the amino terminus and were localized in transfected COS-7 cells using the monoclonal antibody 12C5A5 as described before (von Zastrow and Kobilka, 1992).

RESULTS AND DISCUSSION

We recently reported that when Asn312 in the seventh hydrophobic segment of the β2 receptor is replaced by Phe (β2N312 → F), the amino acid found in the homologous region of the α2 receptor, there is a complete loss of function (Suryanarayana et al., 1991). Not only did this mutant receptor fail to bind agonist and antagonist ligands, but immunocytochemical localization in transfected cells revealed that, unlike wild-type α2 and β2 receptors, β2N312 → F was not delivered to the plasma membrane and was retained in a reticular intracellular compartment (Fig. 1, A and B). These results suggest that a structural incompatibility exists between Phe at position 312 and an adjacent structural domain in β2N312 → F, the amino acid sequence of which is different in α2 and β2 receptors (Fig. 2). In Fig. 2, panel A shows the wild-type β2
**Fig. 1. Immunocytochemical localization of wild-type β2 (A), β2N312 → F (B), and CRS11N312 → F (C) receptors expressed in COS-7 cells.** The receptor proteins were localized by indirect immunofluorescence using a rabbit antiserum directed to the carboxyl terminus of the β receptor as primary antibody and a Texas Red-conjugated goat anti-rabbit IgG as secondary antibody as previously described (Suryanarayana et al., 1991). Specimens were imaged using a Noran Odyssey confocal microscope, with the plane of focus adjusted 2 μm above the surface of the coverslip. The patchy distribution of receptor antigen for wild-type β2 and CRS11N312 → F is probably due to irregularities in the cell surface, which result in different amounts of cell membrane being imaged in the thin optical sections. Although the fluorescent image of CRS11N312 → F (C) is less intense than the image of the wild-type receptor (A), we have not noticed any significant difference in expression of these receptors as determined by ligand binding within a given experiment. Variations in intensity of fluorescence could be due to variability in expression in individual COS cells or bleaching of the fluorophore prior to photography.

**Fig. 2. Models proposed for the interaction of hydrophobic segments in the β2 adrenergic receptor and α2/β2 chimeric receptors.** The hydrophobic segments are shown as viewed from the surface of the cell. Previously published results suggest that the amino nitrogen of the agonist interacts with the Asp13 in the third hydrophobic segment and the two catechol hydroxyls may form hydrogen bonds with Ser117 and Ser119 in the fifth hydrophobic segment of the β2 adrenergic receptor (Strader et al., 1987, 1989) (A). The results presented in this report suggest that the substitution of Phe for Asn112 prevents the seventh hydrophobic segment from interacting with an adjacent structural domain (B) resulting in retention of α2F312 → N in an intracellular compartment and loss of ligand binding ability. After substituting hydrophobic segments 1 and 2 from α2 receptor into this mutant receptor (C-E), its ligand binding properties were restored and it was localized to the plasma membrane. Of the three possible modes of interaction (C-E), the data presented in this report supports the model in which Phe in the seventh hydrophobic segment forms a specific interaction with the amino acid residues in hydrophobic segment 1 (D).

receptor with the agonist epinephrine interacting primarily with hydrophobic segments 3 and 5 (Strader et al., 1987, 1989). In β2N312 → F (Fig. 2B), we propose that the Phe at position 312 is incompatible with adjacent hydrophobic segments. This incompatibility must be great enough to prevent proper receptor folding, thereby abolishing ligand binding and resulting in retention of the misfolded protein in the endoplasmic reticulum (Hurley and Helenius, 1989). If this hypothesis is correct, it should be possible to improve the folding of this β2 receptor mutant by replacing the structural domain adjacent to the Phe312 in β2N312 → F with sequence from the α2 receptor.

We searched for such an interacting domain by constructing a series of chimeric receptors based on the β2N312 → F mutation and containing different hydrophobic segments derived from the α2 receptor (Fig. 3). We found that replacing the first two hydrophobic segments of the mutant receptor with corresponding α2 receptor sequence (CRS11N312 → F) resulted in restoration of plasma membrane localization (Fig. 1C) and recovery of both agonist and antagonist binding (Fig. 3). The fact that such a complementary mutation rescues receptor function supports the hypothesis of incompatibility between adjacent structural domains and suggests more specifically the nature of these interactions (Fig. 2). Our results with CRS11N312 → F suggest several possibilities: Phe312 is interacting with amino acids in both hydrophobic segments 1 and 2 (Fig. 2C), with hydrophobic segment 1 alone (Fig. 2D), or with hydrophobic segment 2 alone (Fig. 2E). The data presented below support the model shown in Fig. 2D.

As shown in Fig. 3, replacing individually the first (CRS13N312 → N) or second (CRS16N312 → N) hydrophobic segments of β2N312 → F with α2 sequence failed to restore function. These results suggest that both hydrophobic segments 1 and 2 are needed to complement the N312 → F mutation (Fig. 2C). However, the failure of the first hydrophobic segment of α2 to restore function in CRS13N312 → F is probably due to the creation of a new structural incompatibility between the first hydrophobic segment of α2 and the second hydrophobic segment of the β2 adrenergic receptor, since CRS13 (which does not contain the inactivating N312 → F mutation) is nonfunctional and is not targeted to the plasma membrane (Fig. 3). Therefore, a specific interaction between Phe312 and only the first hydrophobic domain in CRS11N312 → F (Fig. 2D) remains a possibility. In contrast, substitution of the second hydrophobic segment of the α2 receptor in the

1 It is of interest that CRS11N312 → F has a higher affinity for the α2 receptor antagonist yohimbine (Kd = 35.6 nM) and rauwolscine (Kd = 4.4 nM) than for the β receptor antagonist alprenolol (Kd = 330 nM) (Fig. 3). A similar receptor lacking the N312 → F mutation has a higher affinity for β receptor antagonists (Kobilka et al., 1988).
$\beta_2$ to create CRS16 does not significantly disrupt function (Fig. 3); however, it does not complement the N→F mutation as CRS16N→F is nonfunctional. This result excludes a specific interaction between Phe412 and the second hydrophobic domain alone in CRS11N→F (Fig. 2E).

The results of studies based on complementing the βN→F mutation do not allow us to distinguish between the structural models shown in Fig. 2 (C and D); however, a specific interaction between the seventh hydrophobic segment and the first hydrophobic segment is supported by studies of the converse mutation in the α2 receptor. We have previously reported that changing Phe16 in the seventh hydrophobic segment of the human α2 receptor to Asn (α2F412→N), which is found in the homologous position of the β2 receptor, led to a 300–3000-fold increase in affinity for a class of β receptor antagonists represented by alprenolol (Suryanarayana et al., 1991) (Fig. 3). This mutation also led to 200-fold loss of affinity for epinephrine, an agonist active at both α2 and β2 receptors. Although the mutated amino acid may be directly involved in agonist binding, the results from experiments on the β2N→F mutation presented above suggest that the decreased agonist binding affinity is due to more general conformational changes resulting from incompatibility between Asn at position 412 of α2F412→N and an adjacent structural domain, the amino acid sequence of which is different in α2 and β2 receptors. If this hypothesis is correct, it should be possible to improve the affinity of α2F412→N for agonists by replacing the structural domain adjacent to the Asn412 with sequence from the $\beta_2$ receptor.

The results presented in Fig. 3 indicate that exchanging only the first hydrophobic segment with the corresponding $\beta_2$ receptor sequence can complement the F→N mutation in α2F412→N. CRS6P412→N has more than 10-fold higher affinity for epinephrine than does α2F412→N. For comparison, CRS2P412→N, which has more $\beta_2$ sequence than CRS6P412→N, but does not contain the first or second hydrophobic segments from $\beta_2$, did not show an increased affinity for epinephrine (Fig. 3). These results are consistent with our studies on the β2N412→F mutation and support the model presented in Fig. 2D.

In conclusion, the results of these experiments in which functional abnormalities resulting from a mutation in the seventh hydrophobic segment are complemented by sequence from the first hydrophobic segment are consistent with a structural model in which Asn412 of the $\beta_2$ receptor and Phe412 of the α2 receptor form important structural interactions with the first hydrophobic segment. The arrangement of transmembrane segments predicted by these experiments is similar to that found in bacteriorhodopsin (Henderson et al., 1990). This approach should facilitate the formulation and testing of more accurate models for this class of membrane proteins.

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REFERENCES


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Intramolecular Interactions in Adrenergic Receptors