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REVIEW ARTICLE

In vitro mutagenesis and the search for structure–function relationships among G protein-coupled receptors

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INTRODUCTION: G PROTEIN-LINKED RECEPTORS ARE MEMBERS OF A GENE SUPERFAMILY

A large number of neurotransmitters, peptide hormones, neuromodulators, and autocrine and paracrine factors elicit changes in cellular metabolism by interaction with cell membrane receptors that are coupled to intracellular effector enzymes by guanine-nucleotide-binding regulatory proteins (G proteins) (see Table 1). While the number of endogenous signalling agents that bind to G protein-coupled receptors is quite large, the number of distinct receptors that mediate their actions is even larger. Among neurotransmitter receptors there exist at least ten types of adrenergic receptors (α_{1A}, α_{1B}, α_{1C}, α_{2A}, α_{2B}, α_{2C}, α_{2D}, β_1, β_2 and β_3), at least five types of muscarinic acetylcholine (m_1–m_5) and dopamine (D_1–D_5) receptors, and several serotonergic, purinergic, light (rhodopsin) and olfactory receptors. Similarly, among polypeptide hormone receptors the existence of receptor subtypes is well documented.

G protein-mediated transmembrane signalling pathways have generated a great deal of attention because of the many physiological and pharmacological events that are modulated by these mechanisms. G proteins, which are heterotrimeric proteins composed of α, β, and γ subunits, are members of a large gene superfamily (Gilman, 1987; Iyengar & Birnbaumer, 1990). In the basal state, the G protein oligomer exists in a complex with GDP; the rate of GDP dissociation from the G protein is extremely slow. Following agonist binding, G protein-coupled receptors undergo one or more conformational changes that trigger receptor–G-protein interactions, facilitating an exchange of GTP for bound GDP at a site within the α subunit of the G protein. The binding of GTP to the α subunit of the G protein promotes dissociation of this subunit from the β and γ subunits. GTP-liganded α subunits (and in some cases, the $\beta\gamma$ subunits) are responsible for modulating the activity of distinct effector systems, including adenylate cyclase, phospholipases, cyclic GMP phosphodiesterase and ion channels, leading to metabolic and/or ionic changes within the cell. This reaction is terminated by hydrolysis of bound GTP by a GTPase intrinsic to the α subunit, leading to reassociation of $G\alpha$ with $G\beta\gamma$. The role of the receptor in this system is to serve as a catalyst for the activation of G proteins.

During the past 5 years, more than 100 G protein-coupled receptor subtypes have been cloned and sequenced. This rapid progress has been based in large part on the conservation of primary structure among G protein-coupled receptors, particularly within families, allowing for isolation of new cDNA and genomic clones by cross-hybridization. In addition, the application of new molecular cloning techniques, such as the PCR, has had a major impact on the isolation of G protein-coupled receptor clones. Sequence identity in the regions of the receptor genes coding for transmembrane domains has allowed probes to be generated that hybridize to other members of the family. PCR was first exploited by Libert *et al.* (1989) to isolate several novel members of the G protein-coupled receptor family, and has subsequently been used to isolate cDNAs for the NK-1 (Hershey & Krause, 1990), NK-2 (Gerard *et al.*, 1990), D_1 dopamine (Zhou *et al.*, 1990) and histamine H2 (Gantz *et al.*, 1991) receptors, as well as a new subfamily of odorant receptors (Buck & Axel, 1991).

The visual pigments were the first G protein-linked receptors for which sequence data were obtained. In the early 1980s, the complete amino acid sequences for bovine (Ovchinnikov *et al.*, 1982; Hargrave *et al.*, 1983) and ovine (Pappin *et al.*, 1984) rhodopsin were reported and found to display marked similarities. These sequence data were used to design oligonucleotide probes and clone the genes encoding bovine and human opsins (Nathans & Hogness, 1983, 1984). When the gene encoding a mammalian β-adrenergic receptor (Dixon *et al.*, 1986) was subsequently cloned, it was apparent from the deduced sequence that this receptor exhibited a structure similar to that of the rhodopsins, and suggested the existence of a family of signal receptors.

Current models for the secondary and tertiary structure of G protein-linked receptors are based in large part on the known folding patterns of the ancient retinal-linked visual pigment, bacteriorhodopsin, that is found in naturally occurring lattices within the purple membranes of *Halobacterium halobium*. This pigment, which acts as a proton pump, is not linked to any G protein (Khorana, 1988). When analysed by electron microscopy and high resolution electron diffraction, bacteriorhodopsin is seen as seven α-helices, arranged in a bundle perpendicular to the plane of the lipid bilayer (Henderson & Unwin, 1975; Engelman *et al.*, 1980; Henderson *et al.*, 1990). The basic features of this model are depicted in Fig. 1. Mutagenesis data have supported the idea that the helices are oriented with their hydrophobic faces pointing out into the membrane lipids and their hydrophilic faces point in to the active sites on the molecule (see below).

Comparison of the deduced amino acid sequences of G protein-coupled receptors reveals a similar secondary structure: a single polypeptide chain containing six relatively hydrophobic domains plus a seventh region of lower hydrophobicity. These domains display sequence similarity among most receptor classes and marked similarities among receptor subtypes. Since the seven hydrophobic domains in each receptor are similar in size (20–28 amino acids) and of sufficient length to span the lipid bilayer, they have been postulated to form membrane-spanning domains.

Abbreviations used: ICYP, iodocyanopindolol; QNB, quinuclidinyl benzilate; PI, phosphoinositide; PrBCM, *N*-(2-chloroethyl)-*N*-(2′,3′-propyl)-2-aminoethylbenzilate; PGE, prostaglandin E.

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Table 1. Membrane receptors which interact with G proteins

Peptide receptors	Neurotransmitter receptors
Angiotensin*	Adenosine*
Adrenocorticotropin (ACTH)	α-Adrenergic*
Bombesin*	β-Adrenergic*
Cholecystokinin (CCK)	Dopamine*
Choriogonadotropin*	$GABA_B$
Corticotropin-releasing hormone (CRF)	Histamine*
Follicle-stimulating hormone (FSH)	Muscarinic acetylcholine*
Glucagon	Octopamine*
Gonadotropin-releasing hormone (GnRH)	5-Hydroxy-tryptamine (serotonin)*
Growth-hormone-releasing hormone (GRF)	
Kinins (bradykinin, substance P, substance K)*	Sensory systems
Leutinizing hormone (LH)*	Vision (rhodopsins)*
Melanocyte-stimulating hormone (MSH)	Olfaction*
Neurotensin*	Taste
Opiates	
Oxytocin	Other agents
Parathyroid hormone*	C5a anaphylatoxin*
Somatostatin	Cannabinoids*
Thyrotropin (TSH)*	IgE*
Thyrotropin-releasing hormone (TRH)*	*Mas* oncogene*
Vasoactive intestinal polypeptide (VIP)*	fMet-Leu-Phe*
Vasopressin	Platelet-activating factor*
	Prostanoids* (PGEs, leukotrienes)
	Thrombin*

* Indicates that one or more subtypes from this class of receptors have been cloned and sequenced.

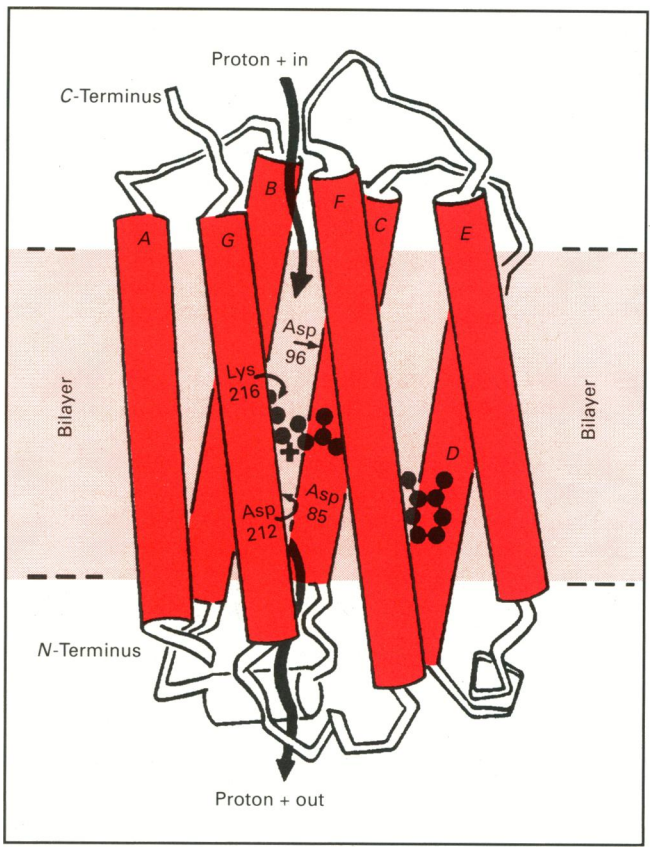

Fig. 1. Tertiary model for bacteriorhodopsin

The tertiary structure is shown of bacteriorhodopsin in the purple membrane obtained by Henderson *et al.* (1990). The transmembrane α-helices (in red) are labelled A–G. Illustrated on the figure are several conserved aspartic acid residues that have been implicated in the proton pumping mechanism of bacteriorhodopsin. Figure reprinted, with permission, from Caspar, D.L.D. (1990) Nature (London) **345**, 666–667 (copyright 1990, Macmillan Magazines Limited).

The intervening sequences, which are considerably more hydrophilic and display greater sequence diversity, form domains that are exposed intracellularly and extracellularly. The *N*-terminal sequence of most G protein-coupled receptors contains putative sites for N-linked glycosylation (Asn-Xaa-Ser/Thr) and is presumed to be located extracellularly (Fig. 2). This model places the *C*-terminus of the receptors intracellularly. Studies on the topography of rhodopsin (Applebury & Hargrave, 1986, and references therein) and the β_2-adrenergic receptor (Wang *et al.*, 1989) support this model for the orientation of G protein receptors in the plasma membrane.

A more detailed comparison of the amino acid sequences of G protein-coupled receptors reveals that they share a number of conserved amino acids or domains (Fig. 3). Some of the more conserved residues among members of this gene family include two extracellular cysteine residues that have been shown to form a disulphide linkage in rhodopsin and the muscarinic receptor (Karnik *et al.*, 1988; Curtis *et al.*, 1989; Kurtenbach *et al.*, 1990), proline residues in helices IV, V, VI and VII, an asparagine in helix I, a leucine in helix II and an arginine in helix III. Atwood *et al.* (1991) noted that conserved polar residues contained within the transmembrane domains were always positioned on the internal side of the helices, and all but one of the conserved aromatic residues were located on external faces of the helices. It was speculated that the strictly conserved residues play an essential role in maintaining the structure of the receptor, perhaps by determining protein folding, whereas those residues conserved only among major classes of receptors may play a role in defining their unique functional properties (Atwood *et al.*, 1991). Conservation of structural and/or functional domains suggests that G protein-coupled receptors may have a common ancestry. It is interesting that genes encoding certain adrenergic receptor subtypes are clustered in the genome (Yang-Feng *et al.*, 1990), suggesting that receptor subtypes may have arisen through gene and/or chromosome duplication.

Since all G protein-linked receptors bind ligands (or in the case of the visual pigments, a chromophore), and activate an appropriate G protein in response to ligand binding, it is not unreasonable to postulate that the receptors have evolved and conserved common structural features related to these shared functions. By utilizing the technique of *in vitro* mutagenesis (see Sambrook *et al.*, 1989, for reviews of methodologies), the role of conserved domains or amino acid residues in receptor function can be studied. Within the limits of the fact that receptors of this gene superfamily bind different ligands and interact with different G proteins, one would hope to define a set of general principles that govern how ligands are bound, how agonists activate a receptor, how receptors interact with G proteins, and how receptor functions are regulated by post-translational modifications. One limitation in studying structure–function relationships of proteins with mutagenesis is that inferences must be made about the role of a particular amino acid(s) based on changes in protein function. Modification of a protein sequence

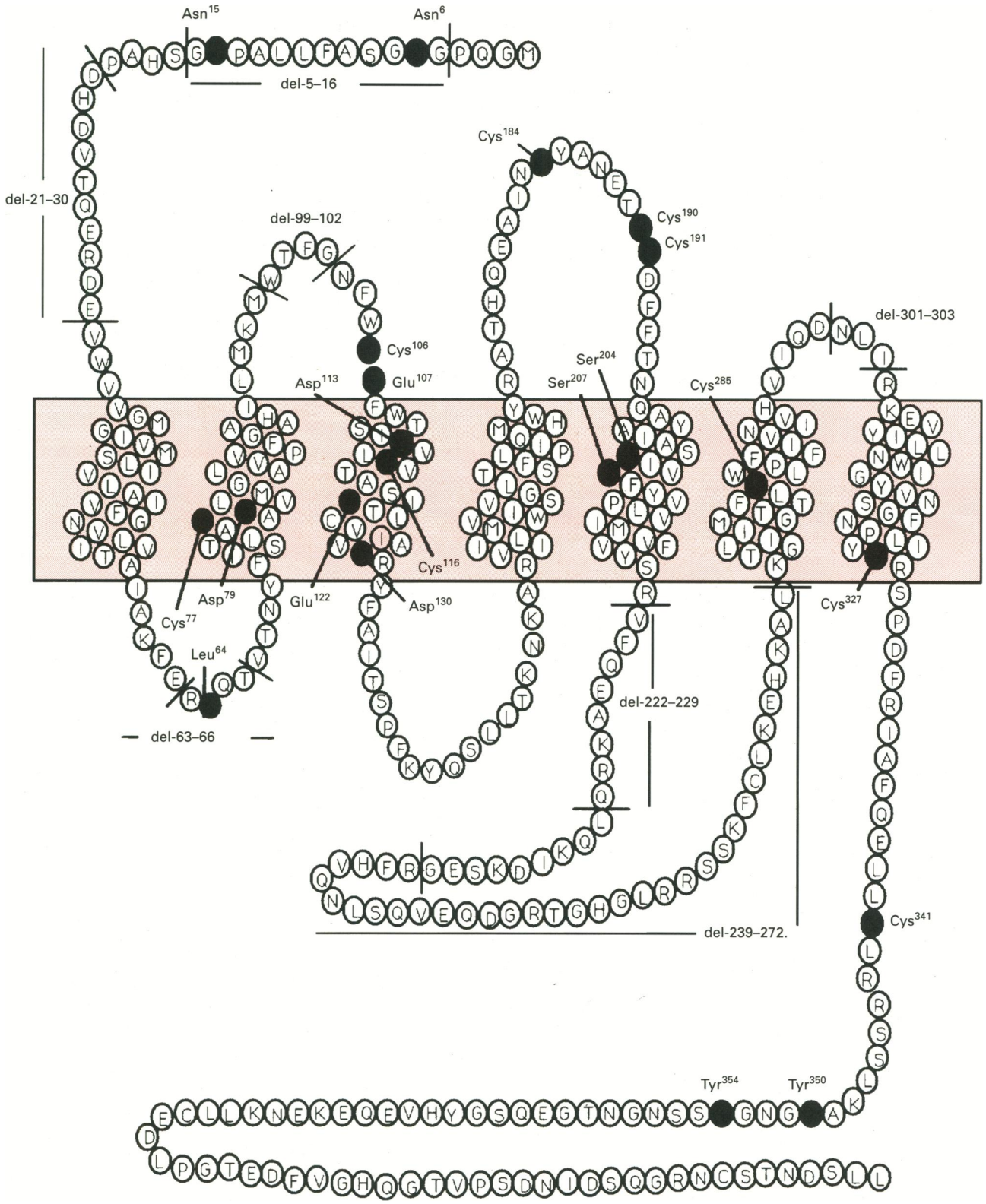

Fig. 2. Mutagenic analysis of the human β_2-adrenergic receptor

Highlighted on this schematic diagram of the human β_2-adrenergic receptor are several key amino acids and domains that have been studied using mutagenesis techniques. The amino acid sequence of the receptor is given by the single letter code. The pink area in the middle of the figure represents the plasma membrane; the areas above and below the membrane represent the extracellular and intracellular space, respectively. The proposed structure of the β_2-adrenergic receptor consists of seven hydrophobic regions spanning the membrane forming extracellular and intracellular loops. The *N*-terminus of the receptor is located extracellularly and the *C*-terminus is located intracellularly.

by mutagenesis may change its biochemical reactivity or produce an unfavourable structural perturbation. Thus one would ideally like to support data obtained from mutagenesis studies with biochemical techniques and vice versa.

The use of *in vitro* mutagenesis to study structure–function relationships in G protein-coupled receptors is relatively recent. It is the purpose of this review to compile and assess the evidence that has been obtained to date. Although there are many gaps in our knowledge, we have tried to emphasize mutagenesis studies on those residues that are present in the majority of G protein-linked receptors, with the hope of identifying some common structure–activity relationships among these proteins. Elucidation of the mechanisms of receptor activation and G protein interactions will continue to be formidable tasks, at least in the near future. In spite of their limitations, mutagenesis studies can at least provide helpful insights into such problems, and can inspire hypotheses that provide directions for continued investigations.

THE SEARCH FOR FEATURES COMMON TO G PROTEIN-COUPLED RECEPTORS

Structural features involved in ligand binding

One of the major functions of most G protein-coupled receptors is to recognize specific hormones/regulatory molecules present in the extracellular environment. The opsins are exceptional is this regard in that their 'ligand', the retinal chromophore, is covalently bound to the apoprotein. Since each receptor–ligand interaction is highly specific, it seems intuitive that each receptor has evolved unique structural elements for ligand recognition.

Much of the evidence obtained to date would suggest that the membrane-spanning regions of G protein-coupled receptors are involved in ligand binding. This concept has its origins in physical studies on bacteriorhodopsin. The all-*trans*-retinal chromophore of bacteriorhodopsin is attached via a Schiff base to Lys^{216}, located in the seventh transmembrane helix of the protein (Bayley *et al.*, 1981; Mullen *et al.*, 1981), and is contained within a pocket formed by the transmembrane helices (Henderson *et al.*, 1990; see Fig. 1). Similarly, the 11-*cis*-retinal chromophore of the visual pigments is covalently attached to Lys^{296} located in transmembrane helix VII of bovine rhodopsin (Wang *et al.*, 1980; Findlay *et al.*, 1981), and is believed to interact with residues on the core-facing surface of one or more helices (Applebury & Hargrave, 1986). It has been speculated that, by analogy, the seven α-helices of G protein-coupled receptors may also form a ligand-binding pocket (Lefkowitz & Caron, 1988; Venter *et al.*, 1989).

Another point relevant to the question of receptor–ligand interactions is that many hormones and neurotransmitters share similar chemical features. For example, many neurotransmitters have a positive charge centre: adrenaline, noradrenaline, dopamine, 5-hydroxytryptamine and histamine each possess an ammonium ion, and acetylcholine contains an onium ion. Also, many peptide hormones, such as the angiotensins, contain basic amino acids that appear to be indispensable for biological activity. This raises the possibility that ligand binding may, in part, involve acidic amino acids. Indeed, several aspartate and glutamate residues are found in transmembrane helices II and III and the intervening first extracellular loop of a number of G protein-coupled receptors (see Fig. 4). Mutagenesis studies (reviewed below) with adrenergic, muscarinic and dopaminergic receptors support a role for these amino acids in receptor–ligand interactions.

1. Adrenergic receptors. (*a*) Identification of ligand-binding domains in β-adrenergic receptors: the importance of conserved aspartate residues. Data from mutagenesis studies have demonstrated that large portions of the extracellular and intracellular hydrophilic regions of mammalian β-adrenergic receptors can be deleted without markedly altering the binding of agonists and antagonists (see Fig. 2 for a diagram of the human β-adrenergic receptor). Dixon *et al.* (1987*a*) deleted segments of the extracellular *N*-terminus of the hamster β_2-adrenergic receptor (del-5–16 and del-21–30) without significantly altering [^{125}I]iodocyanopindolol ([^{125}I]ICYP) or isoprenaline binding. Limited deletions in the extracellular loops between transmembrane regions II and III (del-99–102) and transmembrane regions VI and VII (del-301–303) and also did not affect the binding of isoprenaline to the hamster β_2-adrenergic receptor (Dixon *et al.*, 1987*a*).

Large deletions can also be made in some of the putative cytoplasmic loops and in the *C*-terminus without changing the binding properties of the receptor. Hamster β_2-adrenergic receptors with deletions of residues 229–236 or 239–272, which together represent virtually the entire third cytoplasmic loop connecting transmembrane regions V and VI, display essentially normal ligand binding (Dixon *et al.*, 1987*a*). Several deletions in the first (residues 63–66) or second (residues 130–139, 136–144 or 140–150) putative cytoplasmic loops produce receptors that fail to bind [^{125}I]ICYP, but these mutant receptors could not be detected in immunoblots of membrane preparations using antibodies raised against the *C*-terminus of the receptor, suggesting that the mutant receptors were not being properly processed and/or inserted into membranes (Dixon *et al.*, 1987*b*). The *C*-terminus also appears to play little or no role in ligand binding, as mutations that result in the truncation of large portions of this region in hamster (Dixon *et al.*, 1987*a*) and human (Kobilka *et al.*, 1987*c*) β_2-adrenergic receptors do not alter their binding properties. Taken together, these data suggest that the hydrophilic portions of the β-adrenergic receptor do not play a significant role in ligand binding (Dixon *et al.*, 1987*a*).

A number of lines of evidence implicate the transmembrane regions of β-adrenergic receptors in ligand binding. Peptide mapping of human β_2-adrenergic receptors radiolabelled with the alkylating agent, *p*-(bromoacetamido)benzyl-1-[^{125}I]iodocarazolol identified a 14-amino-acid fragment from transmembrane helix II (residues 83–96) as the site of label incorporation (Dohlman *et al.*, 1988). In a separate study, two β-adrenergic photoaffinity reagents, [^{125}I]-iodocyanopinodolol-diazirine and [^{125}I]iodoazidobenzylpindolol, were used to map the catecholamine-binding domain of the turkey erythrocyte β-adrenergic receptor (Wong *et al.*, 1988). Both of these reagents labelled two sites within the transmembrane helices, one at Trp^{330} in transmembrane helix VII, and a second site mapping to an 8000 Da fragment corresponding to transmembrane regions III, IV and V. Although a reconciliation of these latter findings with those of Dohlman *et al.* (1988) is not immediately apparent, both studies nevertheless support the concept that many, if not all, of the transmembrane helices contribute to the formation of the ligand-binding site.

More recently, physical evidence has been obtained which indicates that the ligand-binding site of the β_2-adrenergic receptor is buried within the lipophilic core of the protein. Tota & Strader (1990) used the fluorescent β-adrenergic antagonist carazolol as a probe to characterize the hamster β_2-adrenergic receptor ligand-binding site. The fluorescence spectrum of the receptor-bound form of this ligand suggested that it was in a hydrophobic environment. Furthermore, agents such as sodium nitrite, which quench the fluorescence of free carazolol, do not quench the receptor-bound form, indicating that the ligand is inaccessible to the solvent, i.e. it is buried within the core of the protein. It was estimated that the carazolol-binding site is 1.1–1.2 nm (11–12 Å)

from the external surface of the receptor, or approximately one-third of the way down the helical core of the receptor (Tota & Strader, 1990).

Evidence has suggested that one or more conserved transmembrane aspartate residues may play a role in catecholamine binding (see Fig. 4). Substitution of Asp^{113} in transmembrane helix III of the hamster β_2-adrenergic receptor with asparagine or glutamate greatly decreased the ability of the receptor to bind [^{125}I]ICYP, dihydroalprenolol and other antagonists (Strader *et al.*, 1987*b*). Further work demonstrated that the $K_{act.}$ of the [Asn^{113}]β_2-receptor for agonist stimulation of adenylate cyclase was increased 8000–40 000-fold relative to the wild-type receptor; substitution of Asp^{113} with glutamate, which also contains a carboxylate side chain, had a less marked effect on receptor activation, increasing the $K_{act.}$ for adenylate cyclase stimulation by agonists 300–1500-fold relative to the wild-type receptor (Strader *et al.*, 1988). Despite these dramatic shifts in $K_{act.}$ values, the mutant receptors retained β_2-adrenergic subtype pharmacology, with a relative agonist potency of isoprenaline > adrenaline > noradrenaline, indicating that site(s) on the receptor other than Asp^{113} are involved in determining the agonist-binding specificities. It was proposed that the carboxylate side chain of Asp^{113} serves as the primary counterion for the cationic amino group of β-adrenergic agonists and antagonists. In a subsequent study, it was shown that the hamster [Glu^{113}]β_2-adrenergic receptor can recognize certain β-adrenergic antagonists such as alprenolol and pindolol as partial agonists (Strader *et al.*, 1989*c*). This phenomenon does not occur in receptors in which an aspartate (i.e. the wild-type receptor) or an asparagine is present at position 113. It was suggested that repositioning of the carboxylate group at this locus as a result of the Asp to Glu substitution might permit the interaction of the aromatic rings of certain antagonists with the agonist-binding site within the receptor (Strader *et al.*, 1989*c*). Wang *et al.* (1991) have also demonstrated that mutation of Asp^{113} in the α_{2A}-adrenergic receptor to Asn markedly reduces agonist and antagonist binding affinities, suggesting that this amino acid may play a similar role in ligand binding in α- and β-adrenergic receptors.

Asp^{79}, a conserved aspartate located in transmembrane helix II, was also found to influence the binding of adrenergic agonists in both hamster (Strader *et al.*, 1987*b*, 1988) and human (Chung *et al.*, 1988) β_2-adrenergic receptors. Substitution of Asp^{79} by alanine in the hamster receptor results in a mutant receptor whose K_d for isoprenaline is increased 10-fold; there is a corresponding 10-fold increase in the $K_{act.}$ of the receptor for adenylate cyclase stimulation in response to this agonist (Strader *et al.*, 1987*b*, 1988). However, antagonist binding was not altered by this substitution. Similarly, when Asp^{79} of the human β_2-adrenergic receptor was replaced by Asn, the resulting mutant receptor displayed significantly decreased affinities for isoprenaline (40-fold), adrenaline (140-fold) and noradrenaline (240-fold); again, antagonist binding [e.g. (−) and (+)-propranolol] was not affected by this mutation (Chung *et al.*, 1988).

The above data implicate transmembrane aspartate groups in ligand binding to β-adrenergic receptors and also impinge on a long standing issue in adrenergic receptor pharmacology: do β-adrenergic antagonists and agonists bind to identical sites on the receptor? The finding that substitution of Asp^{79} with uncharged amino acids in either hamster or human β_2-adrenergic receptors alters the affinity constants for agonists without affecting those for antagonists argues against the view that antagonists and agonists have identical binding sites (Strader *et al.*, 1988; Chung *et al.*, 1988). Nevertheless, mutation of Asp^{113} adversely affects the binding of both classes of ligands, suggesting that the binding sites for agonists and antagonists may overlap (Strader *et al.*, 1988). Similar findings from mutagenesis studies on α_2-adrenergic receptors (Wang *et al.*, 1991), m_1 muscarinic receptors (Fraser *et al.*, 1989*b*) and D_2 dopamine receptors (Neve *et al.*, 1991) suggest that agonist- and antagonist-binding sites in many, if not all, G protein receptors may not be identical.

(*b*) Identification of ligand binding domains in adrenergic receptors: the importance of conserved serine residues. Additional evidence that transmembrane regions of the β_2-adrenergic receptor may be involved in catecholamine binding has been obtained by Strader *et al.* (1989*a*,*b*). Two serine residues at positions 204 and 207 in transmembrane helix V of the hamster β_2-adrenergic receptor have been implicated as hydrogen bonding sites for the catechol hydroxyl groups of β-adrenergic agonists. Conversion of either of these serines to alanines produces receptors with 25–35-fold lower affinity for adrenaline and isoprenaline, but with no change in affinity for β-adrenergic antagonists that lack catechol hydroxyl moieties. Each of these mutant receptors also displays an approx. 50% decrease in their ability to activate adenylate cyclase in response to isoprenaline when expressed in L cells (Strader *et al.*, 1989*a*,*b*). Based on structure–activity studies with a number of isoprenaline analogues, it was postulated that Ser^{204} may form a hydrogen bond with the *m*-hydroxyl substituent of the catechol ring, whereas Ser^{207} may hydrogen bond with the *p*-hydroxyl group. It has been pointed out that conserved serines at these loci are found in all G protein-linked receptors sequenced to date whose ligands possess a catechol ring, e.g. all adrenergic and dopamine receptors, but are not found in those receptors (e.g. muscarinic acetylcholine receptors) whose ligands do not have a catechol ring (Strader *et al.*, 1989*b*).

Because catecholamines serve as endogenous ligands for both α- and β-adrenergic receptors, it is useful to examine whether the serine residues implicated in β-adrenergic receptor–ligand interactions also play a role in α-adrenergic receptor function. Wang *et al.* (1991) constructed mutant α_{2A}-adrenergic receptors in which Ser^{200} or Ser^{204} (which correspond to Ser^{204} and Ser^{207} respectively in the β_2-adrenergic receptor) was replaced with alanine. Characterization of the mutant α_2-receptors suggests that Ser^{204} may participate in hydrogen bond interactions with the *p*-hydroxyl group of the catecholamine ring, as described for Ser^{207} in the β-receptor. However, analysis of mutant [Ala^{200}]α_2-adrenergic receptors suggests that this residue does not directly participate in receptor–agonist interactions. The difference in the reactivity of conserved serine residues in the fifth transmembrane helix of α- and β-adrenergic receptors may be due to the fact that in the β_2-receptor, Ser^{204} and Ser^{207} are located three positions apart, as compared with a distance of four residues apart for Ser^{200} and Ser^{204} in the α_2-adrenergic receptor. Since one turn of an α-helix contains 3.6 amino acid residues, the side chains of Ser^{204} and Ser^{207} in the β_2-receptor and of Ser^{200} and Ser^{204} in the α_2-receptor would assume different orientations in relation to each other that may, in turn, affect their interactions with the catechol ring. These data suggest that although α- and β-adrenergic receptors share common structural domains, the molecular interactions between catecholamines and α-adrenergic and β-adrenergic receptors within this region may not be identical. These findings may, in part, underlie the known pharmacological differences between α- and β-receptors.

(*c*) Identification of domains in β-adrenergic receptors that determine receptor subtype ligand binding specificity. An additional question that has been addressed using mutagenesis techniques relates to the domain(s) responsible for the relative ligand selectivities of β_1-adrenergic versus β_2-adrenergic receptors. A chimeric receptor containing β_2 receptor sequence from the *N*-terminus to the middle of the second cytoplasmic loop (i.e. encompassing transmembrane regions I–III) and β_1

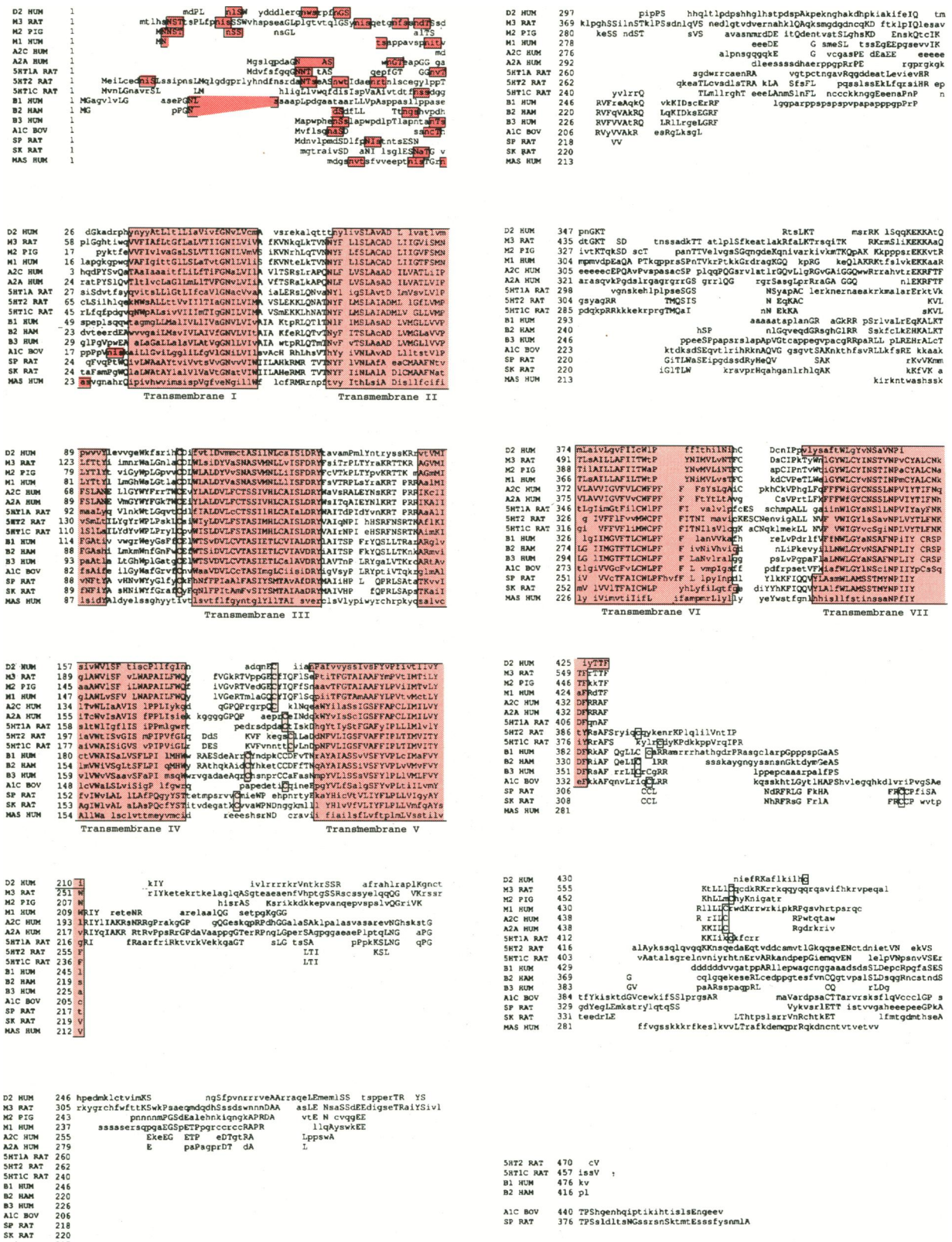

Fig. 3. Protein sequence alignments of representative G protein-coupled receptors

Sequences illustrated are: human D_2 dopamine receptor (D2 HUM; Grandy *et al.*, 1989); rat m_3 muscarinic receptor (M3 RAT; Bonner *et al.*, 1987); porcine m_2 muscarinic receptor (M2 PIG; Kubo *et al.*, 1986*a*; Peralta *et al.*, 1987*a*); human m_1 muscarinic receptor (M1 HUM; Peralta *et al.*, 1987*b*); human α_{2C}-adrenergic receptor (A2C HUM; Lomasney *et al.*, 1990); human α_{2A}-adrenergic receptor (A2A HUM; Fraser *et al.*, 1989*a*); rat 5HT1$_A$ receptor (5HT1A RAT; Albert *et al.*, 1990); rat 5HT$_2$ receptor (5HT2 RAT; Pritchett *et al.*, 1988); rat 5HT1$_C$ receptor (5HT1C RAT; Julius *et al.*, 1988); human β_1-adrenergic receptor (B1 HUM; Frielle *et al.*, 1987); human β_2-adrenergic receptor (B2 HUM; Chung *et al.*, 1987; Kobilka *et al.*, 1987*a*); human β_3-adrenergic receptor (B3 HUM; Emorine *et al.*, 1989); bovine α_{1C}-adrenergic receptor (A1C BOV; Schwinn *et al.*, 1990); rat substance P receptor (SP RAT; Yokota *et al.*, 1989); rat substance K receptor (SK RAT; Sasai & Nakanishi, 1989); human *mas*

receptor sequence for the rest of the protein retained a relative agonist affinity profile similar to that of wild-type β_1-adrenergic receptors, i.e. isoprenaline > adrenaline = noradrenaline (Frielle *et al.*, 1988). However, a chimera containing β_2 receptor sequence from the *N*-terminus to the middle of the second extracellular loop (i.e. encompassing transmembrane regions I–IV), with the remainder of the sequence from the β_1-adrenergic receptor, displayed a relative agonist affinity profile similar to that of the wild-type β_2-adrenergic receptor, i.e. isoprenaline > adrenaline > noradrenaline. These findings suggested that the amino acids of transmembrane helix IV determine the β-adrenergic subtype specificity for agonists (Frielle *et al.*, 1988). This postulate would be greatly strengthened if substitution of transmembrane helix IV from the β_1-receptor with that from the β_2-receptor could by itself confer a β_2-like agonist specificity upon β_1-adrenergic receptors.

2. Muscarinic acetylcholine receptors. Compared with the β-adrenergic receptor, less is known about the specific ligand-binding domain(s) of muscarinic acetylcholine receptors. However, two lines of evidence suggest that conserved aspartates play a role in muscarinic ligand binding in much the same way as they do in β-adrenergic receptors (see Fig. 4).

Site-directed mutagenesis of the rat m_1 muscarinic acetylcholine receptor demonstrated that substitution of Asp^{105} with asparagine produced a receptor that failed to bind the muscarinic antagonist [^{3}H]quinuclidinyl benzilate (QNB) (Fraser *et al.*, 1989*b*). Furthermore, the Asn^{105} m_1 muscarinic acetylcholine receptor was able to activate carbachol-induced membrane phosphoinositide (PI) hydrolysis at a rate of only 1 % that of the wild-type m_1 receptor. These data are consistent with a role for Asp^{105} as a binding site for cationic amines in muscarinic agonists and antagonists, similar to the role that Asp^{113} plays in the β_2-adrenergic receptor.

This idea was confirmed in work from Hulme's laboratory (Curtis *et al.*, 1989; Kurtenbach *et al.*, 1990) using *N*-(2-chloroethyl)-*N*-(2′,3′-[$^3H_2$]propyl)-2-aminoethylbenzilate ([^{3}H]-PrBCM) as an affinity label to identify regions of the muscarinic acetylcholine receptor responsible for binding muscarinic antagonists. Since the aziridine portion of [^{3}H]PrBCM corresponds to the onium group of muscarinic ligands and undergoes attack by nucleophilic amino acid residues within the receptor, this affinity reagent should theoretically label the residue(s) that act as the counterion(s) for the onium ion of muscarinic agents. Rat brain muscarinic acetylcholine receptors (primarily of the m_1 subtype) were isolated, labelled with [^{3}H]PrBCM and subjected to proteolysis using a lysine-specific proteinase and cyanogen bromide; the resulting peptides were sequenced by Edman degradation. The results indicated that [^{3}H]PrBCM labelled Asp^{105} in transmembrane helix III of the rat m_1 muscarinic acetylcholine receptor (Kurtenbach *et al.*, 1990). The Asp^{105} locus is significant in that it aligns with Asp^{113} of the β_2-adrenergic receptor, the putative counterion for the amine function of catecholamines (see above). Thus the aspartate residue in this position appears to serve the same function of cationic ligand recognition in both adrenergic and muscarinic subclasses of receptors.

Asp^{99} (second extracellular domain, aligning with Glu^{107} of the β_2-adrenergic receptor) may also influence ligand binding in the rat m_1 muscarinic acetylcholine receptor. Hulme *et al.* (1990) have reported that this site appears to undergo a limited degree of [^{3}H]PrBCM alkylation. Conversion of this residue to asparagine generated a mutant receptor that displayed 3–5-fold decreases in ligand-binding affinities. In addition, the $[Asn^{99}]m_1$-receptor is labelled by [^{3}H]PrBCM at a level only 10–15 % of that of the wild-type receptor, suggesting that this residue may play a secondary role in the binding of muscarinic ligands (Fraser *et al.*, 1989*b*). Concerning this issue, Kurtenbach *et al.* (1990) have suggested that Asp^{99} may act to stabilize the transition state of alkylation of Asp^{105} by [^{3}H]PrBCM.

3. Thrombin receptors. Evidence that the *N*-terminus of the thrombin receptor is the ligand-binding locus. The previous discussion indicates that the ligand-binding site, in at least some G protein-linked receptors, is located primarily within the transmembrane domains. The thrombin receptor, however, apparently represents a novel paradigm. In an elegant set of studies, Vu *et al.* (1991*a*) isolated a cDNA encoding the human thrombin receptor by expression cloning in *Xenopus* oocytes. Sequence analysis revealed the presence of a thrombin cleavage site, Leu-Asp-Pro-Arg-/-Ser, in the extracellular *N*-terminus of the receptor, 41 amino acids from the initial methionine of the protein. When Arg^{41} was substituted with alanine, the mutant receptor failed to respond to thrombin when expressed in the oocytes. Similar results were obtained when Ser^{42} was substituted with proline, which created a cleavage-resistant Arg-Pro peptide bond. These findings suggested that cleavage of the Leu-Asp-Pro-Arg-Ser site by thrombin may be necessary for receptor activation. A synthetic peptide, representing a portion of the original *N*-terminus of the receptor released upon thrombin-catalysed proteolysis, failed to stimulate the receptor; however, a synthetic peptide, Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe, which mimics the new *N*-terminus created by thrombin action, acted as a potent thrombin receptor agonist. Significantly, this peptide was equally efficacious in activating the cleavage-resistant mutant thrombin receptors containing Ala^{41} and Pro^{42}. It has been proposed that thrombin recognizes the Leu-Asp-Pro-Arg-Ser sequence and that cleavage of the Arg-Ser peptide bond generates a new *N*-terminus which acts as a 'tethered' ligand to trigger receptor activation (Vu *et al.* 1991*a*). Follow-up studies demonstrated that a highly anionic domain located within the *N*-terminus and distal to the thrombin cleavage site participates in the recognition of thrombin, as deletion of this region (positions 51–63) caused a 100-fold shift to the right in the ability of thrombin to trigger receptor activation (Vu *et al.*, 1991*b*).

Evidence for a critical disulphide bond between the putative second and third extracellular domains of G protein-linked receptors

Because of the evidence that has accumulated to implicate disulphide and thiol groups in ligand binding and agonist activation, especially in adrenergic and muscarinic receptors (Lucas *et al.*, 1978; Vauquelin *et al.*, 1979; Aronstam & Eldefrawi, 1979; Abd-Elfattah & Shamoo, 1981; Pederson & Ross, 1985; Moxham *et al.*, 1988, Berstein *et al.*, 1988), cysteine residues have been the subject of considerable investigation using mutagenesis and biochemical techniques. The majority of G protein-coupled receptors sequenced to date contain a pair of conserved cysteine residues in the second and third extracellular domains (see Fig. 3), suggesting the possibility that these cysteines may form a disulphide bond. Other cysteine residues in G protein-coupled receptors display conservation among receptor classes or subtypes.

1. Rhodopsin. There is strong evidence that an essential

oncogene (MAS HUM; Young *et al.*, 1986). Putative transmembrane domains are boxed and labelled. Conserved sites for N-linked glycosylation in the *N*-terminal regions are indicated (N-X-S/T); conserved cysteine residues in the II–III and IV–V extracellular loops are indicated and conserved sites for palmitoylation in the *C*-terminal regions are indicated. Amino acid numbers are shown to the right of the receptor titles.

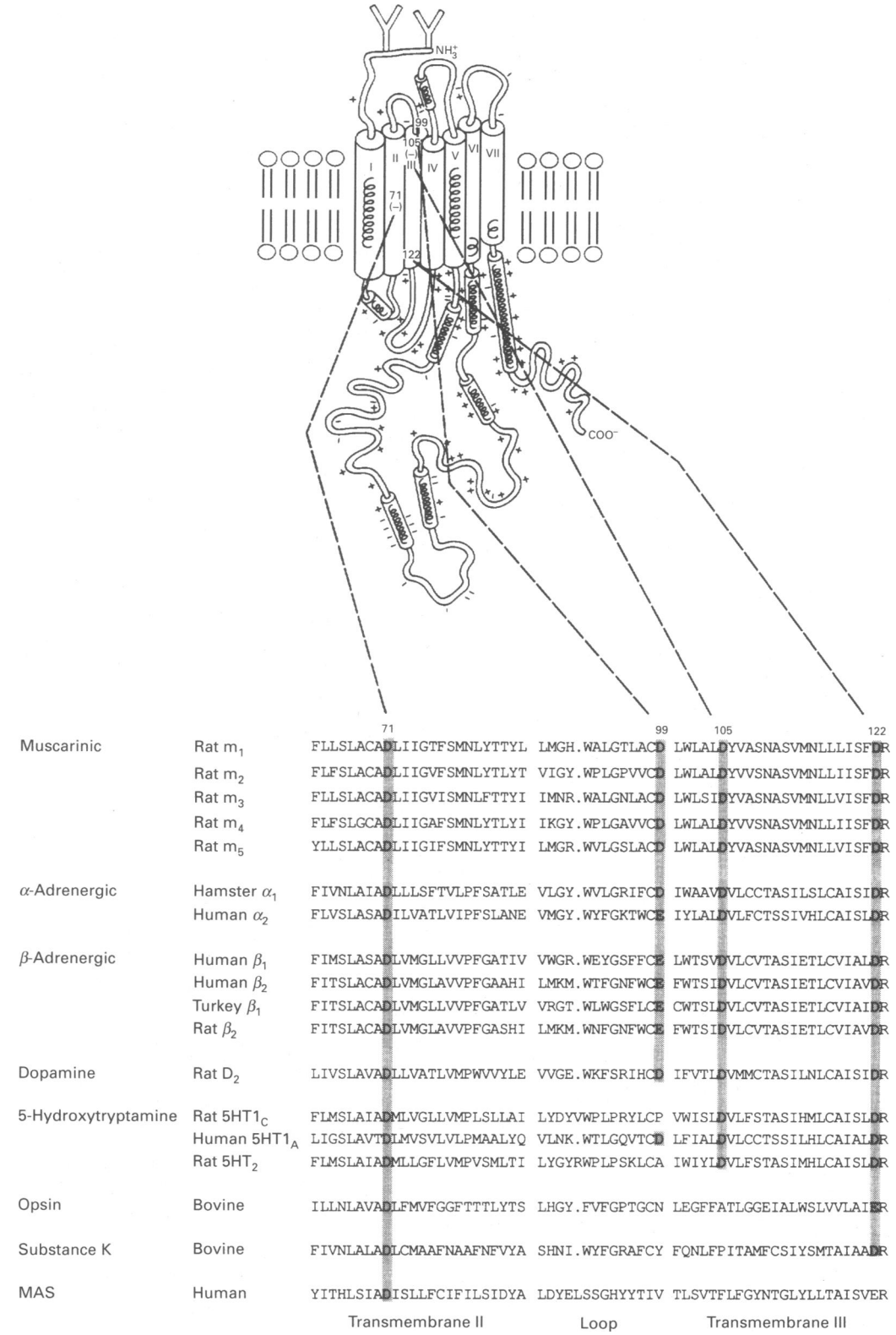

Fig. 4. Conservation of aspartate residues in the second and third transmembrane domains and intervening extracellular loop among members of the G protein-linked receptor supergene family

The numbering and putative locations of these conserved aspartates are depicted using a model of the rat m_1 muscarinic acetylcholine receptor. Note the putative sites of N-glycosylation within the extracellular amino terminus (Y), the regions of α-helices (coils) and the sites of charged residues (− or +). The sequence data are from the following sources: rat m_1–m_5 muscarinic receptors (Kubo *et al.*, 1986*a*,*b*; Gocayne *et al.*, 1987; Bonner *et al.*, 1987, 1988; Liao *et al.*, 1989); hamster α_1-adrenergic receptor (Cottechia *et al.*, 1988); human α_2-adrenergic receptor (Kobilka *et al.*, 1987*b*); human $\beta_1$adrenergic receptor (Frielle *et al.*, 1987); human β_2-adrenergic receptor (Chung *et al.*, 1987; Kobilka *et al.*, 1987*a*); turkey β-adrenergic receptor (Yarden *et al.*, 1986); rat D_2 dopamine receptor (Bunzow *et al.*, 1988); rat $5HT1_C$ 5-hydroxytryptamine receptor (Julius *et al.*, 1988); human $5HT1_A$ 5-hydroxytryptamine receptor (Fargin *et al.*, 1988); rat $5HT_2$ 5-hydroxytryptamine receptor (Pritchett *et al.*, 1988); bovine opsin (Nathans & Hogness, 1983); bovine substance K receptor (Masu *et al.*, 1987); *mas* oncogene (Young *et al.*, 1986).

disulphide bond exists between the second and third extracellular loops of bovine rhodopsin. Early studies on papain-generated peptide fragments of this visual pigment under reducing versus non-reducing conditions indicated the presence of at least one intramolecular disulphide linkage (Sale *et al.*, 1977). Later work by Khorana and colleagues demonstrated that conversion of either Cys^{110} (second extracellular domain) or Cys^{187} (third extracellular domain) to serine produced proteins that do not bind 11-*cis*-retinal, display abnormal glycosylation patterns, and are processed and inserted into membranes at relatively low levels (Karnik *et al*, 1988). It was concluded that in the absence of this putative disulphide bond, rhodopsin does not undergo proper protein folding. Single substitutions of other conserved cysteine residues within bovine rhodopsin, including Cys^{185} (in the second extracellular loop), Cys^{140}, Cys^{167}, Cys^{222} and Cys^{264} (within the transmembrane helices), and Cys^{316}, Cys^{322} and Cys^{323} (within the cytoplasmic carboxyl tail) produce proteins with properties comparable with wild-type rhodopsin (Karnik *et al.*, 1988). A follow-up study employing various combinations of cysteine-to-serine mutations within the protein indicated that the disulphide bond was between the cysteines at positions 110 and 187, and excluded any involvement of the cysteine at position 185; also, it was surmised that the disulphide bond between Cys^{110} and Cys^{187} is apparently buried in rhodopsin, as this disulphide reacted with disulphide reducing agents only upon prior treatment with denaturants (Karnik & Khorana, 1990). The importance of this disulphide linkage in the visual pigments is made clear by the finding that certain individuals with red–green colour blindness carry a hybrid red and green cone pigment gene in which the codon for cysteine at position 184 (corresponding to Cys^{187} in bovine rhodopsin) is substituted with an arginine codon; thus the absence of a critical disulphide bridge in this pigment may be responsible for this abnormal phenotype (Nathans *et al.*, 1989).

2. β-Adrenergic receptors. In the hamster β_2-adrenergic receptor, conversion of either Cys^{106} in the second extracellular domain or Cys^{184} in the third extracellular domain (see Fig. 2) to valine causes a complex shift in the agonist-binding properties of the mutant receptors (Dixon *et al.*, 1987*b*). Mutant receptors containing either Val^{106} or Val^{184} both display two classes of binding sites for isoprenaline, one of high and one of low affinity, whereas the wild-type receptor normally exhibits only a single class of sites with intermediate affinity. The fact that the double mutation does not have an additive effect on isoprenaline binding is consistent with the hypothesis that Cys^{106} and Cys^{184} are involved in a disulphide linkage. These findings were confirmed by Dohlmann *et al.* (1990), who converted Cys^{106} and Cys^{184} to valine in the human β-adrenergic receptor.

Two other cystine residues, Cys^{190} and Cys^{191}, located on the third extracellular loop of β-adrenergic receptors, have been shown to influence ligand binding. Fraser (1989) demonstrated that replacement of Cys^{190} or Cys^{191} with serine in the human β_2-adrenergic receptor produced mutant receptors with 8- and 720-fold decreases respectively in their affinity for isoprenaline as compared with the wild-type receptor. Both of these mutant receptors also displayed a marked shift to the right in the dose–response curve for isoprenaline-induced increases in intracellular cyclic AMP concentrations, relative to the normal receptor, in stably transfected B-82 cells. Significantly, K_d values of the double mutant ($Ser^{190,191}$) receptor were higher than those of either single-mutant receptor, suggesting that these cysteines moieties function independently (Fraser, 1989). In confirmation of these findings, Dohlman *et al.* (1990) have reported that conversion of Cys^{190} and Cys^{191} in the human β-adrenergic receptor to valine decreases the receptor affinity for agonists and antagonists. In the studies by Fraser (1989) and Dohlman *et al.* (1990), it was found that mutation of the extracellular cysteine residues in the β-adrenergic receptor markedly reduced levels of receptor expression compared with the wild-type receptor, suggesting that these residues may also be required to maintain the receptor in the correct conformation for processing and membrane insertion.

3. Muscarinic acetylcholine receptors. There is evidence that Cys^{98} and Cys^{178} in the second and third extracellular loops respectively of the rat m_1 muscarinic acetylcholine receptor are involved in a disulphide bridge. In proteolysis studies with rat brain muscarinic receptors labelled with [^{3}H]PrBCM, Curtis *et al.* (1989) observed a 14 kDa labelled peptide product upon lysine protease digestion that was present as a 22 kDa fragment if isolated under non-reducing conditions. This peptide was converted to a 14 kDa product (and an unlabelled 8 kDa fragment) under reducing conditions, suggesting the presence of a disulphide linkage. Based on cleavage sites predicted by the amino acid sequence, these fragments were hypothesized to represent portions of the second and third extracellular loops; it was presumed that Cys^{98} and Cys^{178} were responsible for the disulphide linkage. In a subsequent study, peptide sequencing of purified rat m_1 muscarinic acetylcholine receptor that had been treated with [^{3}H]*N*-ethylmaleimide under conditions designed to promote its incorporation into disulphide-bonded cysteines indicated that at least one site of labelling was Cys^{98} (Kurtenbach *et al.*, 1990).

In site-directed mutagenesis studies, conversion of either Cys^{98} or Cys^{178} to serine generates mutant receptors that display only negligible binding of the muscarinic antagonist [^{3}H]QNB (Savarese *et al.*, 1990). It remains to be determined if these mutations directly affect ligand binding, or alter the processing of the protein and/or its insertion into the plasma membrane. However, it appears that the role of the conserved extracellular cysteines in muscarinic and β-adrenergic receptors may not be identical, as mutant β-receptors lacking either of these residues are transported to the membrane and retain the ability to bind ligands, in contrast to the findings with the muscarinic receptor.

There are examples of G protein-coupled receptors that lack a cysteine residue in the second extracellular loop, including the human *mas* oncogene (Young *et al.*, 1986) which reportedly encodes a receptor for the angiotensins (Jackson *et al.*, 1988) and the rat cannabinoid receptor (Matsuda *et al.*, 1990). It may be that these proteins are members of a subfamily of G protein-linked receptors that diverged from the prototypical neurotransmitter receptor.

Domains involved in receptor activation: role of conserved transmembrane aspartate residues in receptor activation

As an integral part of the process of receptor activation, agonist binding is thought to induce a conformational change in the receptor that facilitates receptor–G-protein interactions. Despite the fact that the processes of receptor activation (agonist-induced conformation shifts within the receptor) and G protein activation are connected, there may be distinct functional sites within the receptor responsible for receptor activation and receptor–G-protein interactions.

1. Aspartate residues and the activation of light receptors. Light-induced activation of bacteriorhodopsin involves ionic movements across the cell membrane through a series of charged residues. Absorption of light by the all-*trans*-retinal chromophore triggers a photochemical cycle in which the chromophore is converted to 13-*cis*-retinal; during this cycle the Schiff base formed by attachment of the retinal to Lys^{216} of bacteriorhodopsin undergoes protonation and deprotonation (Khorana, 1988). This photochemical cycle drives the translocation of protons from inside to outside the cell, creating an electro-

chemical gradient that is utilized by the cell for ATP synthesis (Findlay & Pappin, 1986).

Aspartate residues in the transmembrane domains of bacteriorhodopsin (see Fig. 1) have been shown to play a critical role in the proton pumping process. Site-directed mutagenesis studies (Mogi *et al.*, 1988) have shown that replacement of Asp^{85} or Asp^{96} (both located in transmembrane helix III) by asparagine nearly abolishes the ability of bacteriorhodopsin to translocate protons. Conversion of another aspartate, Asp^{212} (transmembrane helix VII), to either asparagine or glutamate also results in a decreased proton pumping capacity ($\sim$ 10 and 20% respectively of that of wild-type receptors). Together, these data suggest that these three aspartate residues are involved in a proton conductance mechanism in bacteriorhodopsin. This hypothesis is supported by an atomic model of the structure of *Halobacterium halobium* bacteriorhodopsin that has been recently obtained by Henderson *et al.* (1990) using high resolution electron cryomicroscopy.

The role of negatively charged amino acids in the activation mechanism of the G protein-linked mammalian visual pigments is less well understood. It has been speculated that one or more negatively charged amino acids, presumably at or near the putative core of the pigment protein, may serve as a counterion for the 11-*cis*-retinal chromophore, which exists as a protonated Schiff base (Oseroff & Callender, 1974) bound to a lysine moiety within the seventh transmembrane helix (Applebury & Hargrave, 1986). It is this interaction that accounts for the difference in the absorption maximum of the free protonated retinylidene Schiff base in methanol compared with that of rhodopsin, the fact that different visual pigments containing the same chromophore have varying absorption maxima, and is presumably important in the light-induced receptor activation mechanism. Several independent studies employed site-directed mutagenesis of charged amino acid residues located at or near the transmembrane domains of bovine rhodopsin to determine if they serve as the counterion for the protonated chromophore. Two of these studies found that conversion of Glu^{113}, which (depending on the model used) is located in either the second extracellular loop or the third transmembrane domain, to glutamine shifted the absorption maximum of this protein from 500 nm to 380 nm (Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989). Conversion of other potential counterions, such as Asp^{83} (second transmembrane domain), Glu^{122} (third transmembrane domain) and Glu^{134} (third transmembrane domain) to neutral amino acids caused either small or no spectral shifts (Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989; Nathans, 1990). These findings suggested that Glu^{113} is the counterion for the protonated retinylidene Schiff base in bovine rhodopsin (Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989). However, Janssen *et al.* (1990) reported that the Gln^{113} bovine rhodopsin did not display spectral characteristics that were markedly different from those of the native protein; the reason for this discrepancy is not apparent. Another issue that has to be considered is that some invertebrate visual pigments lack a negatively charged amino acid in this position. Further work is needed to clarify the role of individual amino acids of the opsin apoprotein in spectral tuning.

2. Aspartate residues and activation of G protein-coupled receptors. A working hypothesis of agonist-induced receptor activation (Venter *et al.*, 1989) based on site-directed mutagenesis studies, primarily with the human β_2-adrenergic receptor, and the known mechanisms of light activation of bacteriorhodopsin has been proposed. As in bacteriorhodopsin, many G protein-coupled receptors contain a number of conserved carboxylic acid-containing amino acids within transmembrane helices II and III. In the β-adrenergic receptor, for example, Asp^{113} (transmembrane helix III) is near the extracellular surface of the receptor, Asp^{79} (transmembrane helix II) and Glu^{122} (transmembrane helix III) are near the centre of the transmembrane helix, and Asp^{130} (transmembrane helix III) is located near the cytoplasmic surface of the receptor. It has been proposed that in the unliganded receptor, these negatively charged side chains may be complexed to water molecules or to mono- or divalent cations. The conformational change in the receptor following agonist binding to Asp^{113} (see above) may initiate ion transfer across the membrane, in a manner analogous to the relay system that exists in bacteriorhodopsin (see above). This process may in turn alter the conformation of the cytoplasmic domains of the receptors, facilitating receptor–G protein interactions.

Recently, however, Strader *et al.* (1991) have challenged the hypothesis that the aspartate residues of the transmembrane regions of the β_2-adrenergic receptor participate in a charge relay system that triggers receptor activation. They converted Asp^{113}, the presumed counterion for the amine group of the catecholamines, to Ser, and examined a series of modified catechol-containing ketones and esters that lacked an amine function for their ability to activate the Ser^{113} mutant receptor. It was reasoned that these catechol derivatives might form hydrogen bonds with the hydroxyl side chain of Ser^{113} and trigger receptor activation. Indeed, several of these catechol esters and ketones, including those with α- and γ-substitutions, were capable of fully activating the Ser^{113} mutant receptor, in terms of inducing increases in adenylate cyclase activity, but were unable to activate the native β_2-adrenergic receptor containing Asp at position 113. It was argued that if receptor activation could only occur when the ionic interaction between the positively charged amine group of the catecholamine and the negatively charged carboxylate of Asp^{113} is transferred to another acidic residue, then these catechol derivatives which lack amine functions should not have been able to act as full agonists.

Nevertheless, it is clear that the conserved aspartate in the second transmembrane domain of G protein-coupled receptors is of vital importance in the signal transduction mechanism. The amino acid sequences of virtually all receptors in this family contain an aspartate at this locus (Figs. 3 and 4). Mutation of this residue in the human β_2-adrenergic (Asp^{79}; Chung *et al.*, 1988), human α_2-adrenergic (Asp^{79}; Wang *et al.*, 1991), rat D_2 dopamine (Asp^{80}; Neve *et al.*, 1991) and rat m_1 muscarinic acetylcholine (Asp^{71}; Fraser *et al.*, 1989*b*) receptors markedly attenuates (*a*) agonist efficacy for stimulation of adenylate cyclase (β_2-adrenergic receptor), (*b*) attenuation of adenylate cyclase (α_2-adrenergic receptor, D_2 dopamine receptor), and (*c*) activation of phospholipase C (m_1 muscarinic acetylcholine receptor).

This conserved aspartate residue may play a pivotal role in the allosteric actions of monovalent cations on certain G protein-coupled receptors. Monovalent cations such as sodium modulate ligand binding to many members of the G_i-linked subclass of receptors (i.e. those that mediate an inhibition of adenylate cyclase) via an allosteric mechanism. In the case of the α_2-adrenergic receptor, for example, Na^+ ions increase receptor affinity for antagonists such as yohimbine, but decrease affinity for agonists such as adrenaline (Limbird, 1984). When Asp^{79} of the porcine α_2-adrenergic receptor is converted to asparagine, the allosteric effects of Na^+ ions are completely abolished, suggesting that Na^+ ions may bind to the carboxylate moiety of this aspartate to induce the allosteric modulation (Horstman *et al.*, 1990). In another G_i-linked receptor, the rat D_2 dopamine receptor, substitution of Asp^{80} with either alanine or glutamate greatly diminishes the ability of this receptor to inhibit adenylate cyclase activity (Neve *et al.*, 1991). These substitutions also markedly reduce the sensitivity of the D_2 dopamine receptor to Na^+ ions, which in the 100 mM range increase receptor affinity for

substituted benzamide antagonists such as epidepride (Neve *et al.*, 1990). It has been proposed that D_2 receptors undergo a monovalent-cation-dependent isomerization that is important for both ligand affinity and receptor activation, and that the interaction of monovalent cations with Asp^{80} is crucial for these conformational events (Neve *et al.*, 1991).

Domains involved with coupling to G proteins: cytoplasmic domains as potential sites for receptor–G protein interactions

One might predict *a priori* that the cytoplasmic domains of G protein-coupled receptors would be the site(s) of receptor–G protein interactions. Because this family of receptors collectively interacts with a number of distinct G proteins, it seems plausible that the site(s) for receptor–G protein interactions might be located in regions that contain relatively non-similar sequences. The amino acid sequences of the first and second cytoplasmic loops of G protein receptors are relatively conserved, and as such are not attractive as potential sites for determining receptor–G protein specificity (Lefkowitz & Caron, 1988). Instead, attention has focused on the third cytoplasmic loop (between transmembrane helices V and VI) and the *C*-terminus, which display a greater degree of sequence and size heterogeneity among and within these receptor gene families.

However, an argument for the involvement of other cytoplasmic domains of G protein receptors in receptor–G protein coupling can be made, based on theoretical grounds. Weiss *et al.* (1988) have pointed out that although the opsins have a minimal putative third cytoplasmic loop, rhodopsin is capable of activating purified preparations of a variety of G proteins, such as G_i and G_o, in functional reconstitution assays (Tsai *et al.*, 1987). G_i has been shown to associate with α_2-adrenergic and m_2 muscarinic acetylcholine receptors, which possess relatively large third cytoplasmic loops. Thus the implication is that much of the third cytoplasmic loop of adrenergic and muscarinic receptors has little to do with G protein recognition.

1. β-Adrenergic receptors. Several mutants of the hamster β_2-adrenergic receptor with deletions in the putative third cytoplasmic loop have been described that have markedly decreased abilities to stimulate adenylate cyclase. Receptors containing deletions of residues 239–272, representing almost two-thirds of the third cytoplasmic loop (see Fig. 2), were unable to activate adenylate cyclase when expressed in L cells (Dixon *et al.*, 1987*a*). Further studies revealed that the ability of the receptor to stimulate adenylate cyclase could be virtually eliminated by deletion of a relatively short segment in this loop, residues 222–229 (Strader *et al.*, 1987*a*). A third deletion mutant (residues 258–270), had a greatly diminished ability to activate adenylate cyclase (Strader *et al.*, 1987*a*). As evidence that these deletions result in receptors that are uncoupled from G_s, none of these three mutant receptors showed a significant shift to the right in [^{125}I]ICYP/isoprenaline competition binding curves in the presence of the GTP analogue guanosine 5′-[βγ-imido]triphosphate (Strader *et al.*, 1987*a*). In addition, Cheung *et al.* (1989) showed that one of the mutant receptors which does not activate adenylate cyclase, del-222–229, does not undergo sequestration upon prolonged exposure to isoprenaline, whereas mutants that stimulate adenylate cyclase, even with low efficacy, such as del-258–270, are sequestered at rates comparable with that of wild-type β_2-adrenergic receptors. Finally, a mutagenesis study on the human β_2-adrenergic receptor identified a seven-amino-acid segment (residues 267–273) in the *C*-terminal end of the third cytoplasmic loop whose deletion results in a receptor that can activate adenylate cyclase with an efficacy of only 50% (Hausdorff *et al.*, 1990).

Kobilka *et al.* (1988) have attempted to delineate the regions of the human β_2-adrenergic receptor that are responsible for G_s activation by the use of receptor chimeras. These investigations substituted various regions of the human β_2-adrenergic receptor into the sequence for the human α_2-adrenergic receptor, and expressed the chimeras in *Xenopus* oocytes. Since α_2-adrenergic receptors are primarily coupled to G_i, these workers examined a variety of chimeras to see which of these acquired the capacity to stimulate adenylate cyclase, i.e. which of these acquired β_2-adrenergic receptor function. An α_2-adrenergic receptor in which a portion of transmembrane region V, all of transmembrane region VI and the intervening third cytoplasmic loop were replaced with the corresponding sequence of the β_2-adrenergic receptor activated adenylate cyclase with an α_2-adrenergic order of agonist potency and an efficacy approx. one-third that of wild-type β_2-adrenergic receptors. Chimeric receptors that contained only a portion of the third intracellular loop of the β_2-adrenergic receptor lost the ability to stimulate adenylate cyclase. These observations suggested that the entire third cytoplasmic loop of β_2-adrenergic receptors may be required for G_s coupling. However, all cytoplasmic domains of the β_2 receptor were clearly necessary to achieve a maximal stimulation of adenylate cyclase, since the presence of any cytoplasmic sequence from the α_2 receptor markedly decreased the magnitude of this response. These findings illustrate some of the difficulties in interpretation of data obtained with chimeric receptors, because the responses obtained with chimeras are often quantitatively very different from those of either wild-type receptor. Furthermore, it was shown that human α_2-adrenergic receptors can activate adenylate cyclase in response to high concentrations of adrenaline when expressed in CHO cells (Fraser *et al.*, 1989*a*); this observation obfuscates any conclusions that can be drawn about G protein binding sites based on α_2/β_2-adrenergic receptor chimeras.

Asp^{130}, a highly conserved residue located at the interface between transmembrane helix III and the *N*-terminal end of the second intracellular loop in the human β_2-adrenergic receptor (see Fig. 2), appears to be critical for proper G protein coupling. Conversion of this Asp to Asn virtually abolishes the ability of the receptor expressed in B-82 cells to activate adenylate cyclase (Fraser *et al.*, 1988). Because this residue is located within or very near to the cytoplasm, it is tempting to speculate that the presumptive conformation shifts which occur upon receptor activation may expose, or alternatively mask, the carboxylate side chain of Asp^{130}, thus altering the interaction of the receptor with G_s. Consistent with this hypothesis is the finding that when the corresponding residue in bovine rhodopsin, Glu^{134}, is reversed with its neighbouring residue, Arg^{135}, the mutant rhodopsin is unable to bind to transducin (see below; Franke *et al.*, 1990). However, when the analogous aspartate in the rat m_1 muscarinic acetylcholine receptor, Asp^{122}, is substituted with Asn, the efficacy of the mutant receptor for activating PI hydrolysis is not diminished, although agonist potency is reduced (Fraser *et al.*, 1989*b*). A similar shift to the right in the dose–response curves for agonist-mediated inhibition of adenylate cyclase has recently been reported for the Asn^{130} mutant α_2-adrenergic receptor (Wang *et al.*, 1991). Thus this locus may be important for receptor coupling to some, but not all, G proteins.

O'Dowd *et al.* (1988) have described several point mutations in cytoplasmic regions of the human β_2-adrenergic receptor which influence the ability of the mutant receptors expressed in *Xenopus* oocytes to activate adenylate cyclase. Conversion of Leu^{64} in the first cytoplasmic loop to glycine, and Cys^{327} and Cys^{341} in the *C*-terminus to arginine and glycine respectively, reduces the ability of the receptor to activate adenylate cyclase in response to isoprenaline by approx. 60, 70 and 40% respectively. It has been demonstrated that Cys^{341} in the *C*-terminus of the human β_2-adrenergic receptor is thioesterified with palmitic acid (O'Dowd *et al.*, 1989). Substitution of Cys^{341} with glycine produces a non-

palmitoylated receptor that has a markedly decreased ability to activate adenylate cyclase. Based on the earlier work of Ovchinnikov *et al.* (1988), who demonstrated that two adjacent cysteine residues in the *C*-terminus of bovine rhodopsin (Cys^{322} and Cys^{323}) are palmitoylated and speculated that the palmitoyl moieties are embedded in the membrane to form a 14-amino-acid cytoplasmic loop, it was proposed that a similar structure in the β-receptor may play a critical role in receptor–G protein interactions (O'Dowd *et al.*, 1989). It should be noted, however, that although both Cys^{322} and Cys^{323} in bovine rhodopsin are palmitoylated (Ovchinnikov *et al.*, 1988), conversion of both of these residues to serine produced a mutant rhodopsin capable of activating transducin at a level comparable with the wild-type protein (Karnik *et al.*, 1988). A potential consensus sequence for palmitoylation, Leu-(Xaa)-Cys-$(Xaa)_n$-(Arg/Lys)- has been proposed (Strittmatter *et al.*, 1990). This sequence (or portions of it) is found in many, but not all, G protein-coupled receptors, with the critical cysteine residue located ~ 11–16 residues distal to the end of transmembrane helix VII (see Fig. 3). Future studies with other receptors in this family will better elucidate the role of palmitoylation in receptor function.

2. α_1-Adrenergic receptors. Cotecchia *et al.* (1990) have obtained evidence that both the third intracellular loop and the *C*-terminus of the hamster α_1-adrenergic receptor play a role in G protein-mediated coupling of this receptor to phospholipase C. Chimeric receptors in which virtually the entire third intracellular loop (residues 228–295) of the hamster α_1-adrenergic receptor was substituted for the same domain in the human β_2-adrenergic receptor was capable of activating PI hydrolysis at levels approaching that of the native hamster α_1-adrenergic receptor when expressed in COS-7 cells, even though the chimeric receptor retained the ligand-binding characteristics of β_2-adrenergic receptors. This suggested that the third intracellular loop confers G protein coupling specificity in the α_1-adrenergic receptor.

To identify regions within this domain of the α_1-adrenergic receptor that are responsible for G protein interactions, shorter stretches of sequence in this loop were substituted with those from the human β_2-adrenergic receptor. One substitution, in which Thr-Leu-Arg-Ile-His-Ser-Lys-Asn (residues 252–259 of the α_1-adrenergic receptor, near the middle of the third-intracellular loop) was replaced with Glu-Gly-Arg-Phe-His-Val-Gln-Asn, resulted in a receptor whose ability to active PI hydrolysis in response to noradrenaline was reduced by 90%. Unexpectedly, replacement of residues 288–294 in the α_1-adrenergic receptor (Arg-Glu-Lys-Lys-Ala-Ala-Lys, at the *C*-terminal end of the third intracellular loop) with the sequence Lys-Glu-His-Lys-Ala-Leu-Lys produced a receptor that displayed a 100-fold higher affinity for noradrenaline than did the wild-type α_1-adrenergic receptor, and a 300-fold increase in potency for activating PI breakdown. Since this substitution was relatively conservative, involving only three amino acid mutations (Arg^{288} to Lys, Lys^{290} to His and Ala^{293} to Leu), each of these point mutations was analysed individually. Both the His^{290} and Leu^{293} mutant α_1-adrenergic receptors had markedly decreased K_i values for noradrenaline and increased potency for activation of PI turnover relative to the wild-type receptor. These mutants also displayed higher basal inositol phosphate levels, leading to the speculation that these point mutations in some way lock the receptor into its active conformation (Cotecchia *et al.*, 1990).

The *N*-terminal portion of the cytoplasmic tail may also be important for α_1-adrenergic receptor–G protein coupling. Substitution of a 13-amino-acid segment (positions 353–365) of the hamster α_1-adrenergic receptor, including the cysteine residue that may serve as a site of palmitoylation, with the corresponding sequence of the human β_2-adrenergic receptor produced a receptor whose capacity for activating phospholipase C was impaired by 40% (Cotecchia *et al.*, 1990).

3. α_{2A}-Adrenergic receptors. A recent study by Dalman & Neubig (1991) utilized six peptides derived from various cytoplasmic domains of the α_{2A}-adrenergic receptor to probe receptor–G protein interactions. Two peptides from (*a*) the second cytoplasmic loop (peptide A; Gln-Ala-Ile-Glu-Tyr-Asn-Leu-Lys-Arg-Thr-Pro-Arg) and (*b*) the *C*-terminal end of the third cytoplasmic loop (peptide Q; Arg-Trp-Arg-Gln-Arg-Gln-Asn-Arg-Glu-Lys-Arg-Phe-Thr) were potent inhibitors of agonist binding to the receptor; this inhibitory effect was abolished in the presence of guanine nucleotides. Peptide Q also inhibited agonist-stimulated GTPase activity, suggesting that it could mimic the receptor and bind directly to G_i to prevent coupling to the receptor. The interaction of peptide A with the receptor and G protein was distinct from that of peptide Q, as it did not prevent receptor-mediated activation of the G protein. Together, these data suggest that both the second cytoplasmic loop and the *C*-terminal portion of the third cytoplasmic loop of the α_{2A}-receptor are involved in the interaction with G_i.

4. Rhodopsin. Takemoto *et al.* (1985) tested a number of synthetic peptides, 5–9 amino acids in length, corresponding to various putative cytoplasmic regions of bovine rhodopsin, including the *C*-terminal tail and the three cytoplasmic loops, for their ability to inhibit activation of exogenous transducin by rod-outer-segment disc membranes that had been depleted of transducin. Three peptides, corresponding to bovine rhodopsin residues 333–339, 324–331 and 317–321, all located in the *C*-terminus, were able to block transducin activation, as reflected by a decrease in transducin-catalysed GTPase activity. The peptide corresponding to amino acids 333–339 was the most potent inhibitor among these. Peptides corresponding to the last eight residues on the *C*-terminus, and the three cytoplasmic loops, failed to block transducin activation in this system. These investigators concluded that residues 317–339 may be the recognition site on rhodopsin for transducin. This domain, however, does not appear to be highly conserved among the opsins. Furthermore, removal of the last 12 amino acids of the *C*-terminus by proteolysis was shown to have little effect on the ability of transducin to bind to rhodopsin (Kuhn, 1984).

In contrast, Franke *et al.* (1988, 1990) found evidence that both the second and third cytoplasmic loops of bovine rhodopsin may be involved in transducin recognition and activation. These investigators created three mutant bovine rhodopsins that lacked the ability to stimulate light-induced GTPase activity associated with transducin (Franke *et al.*, 1990). The first mutant involved a reversal of the charge pair Glu^{134}/Arg^{135}, located at the *N*-terminal end of the second intracellular loop, to Arg^{134}/Glu^{135}. This mutant failed to bind transducin, suggesting that these residues directly participate in the binding reaction or are essential for the correct conformation of the transducin-binding site. Two other mutant rhodopsins, one in which the sequence Cys-Lys-Pro-Met-Ser-Asn-Phe-Arg-Phe-Gly-Glu-Asn-His within the central region of the second intracellular loop is replaced with Gly-Thr-Glu-Gly-Pro-Asn-Phe-Tyr-Val-Pro-Phe-Thr-Ser, and another in which 13 amino acids from residues 237 to 249 in the middle of the third intracellular loop are deleted, can bind transducin but fail to release this G protein in the presence of GTP. It was speculated that these mutant opsins can trigger the release of bound GDP from transducin, but cannot induce the formation of the GTP-binding site within the G protein, leading to an inactive opsin–transducin complex (Franke *et al.*, 1990). The last mutant is of interest since it corroborates an earlier site-directed mutagenesis study in which Lys^{248} of bovine rhodopsin was substituted with Leu (Franke *et al.*, 1988). This mutation

produced a rhodopsin that was completely unable to activate transducin, as monitored by GTPase activity. A lysine residue equivalent to Lys^{248} has been found in all visual pigments sequenced to date, except for the human blue pigment, which contains an arginine (Nathans *et al.*, 1986). Interestingly, a comparable residue is present among the β-adrenergic receptors. However, if a triple mutant is created in which the two glutamate residues adjacent to Lys^{248} are also mutated to neutral amino acids (i.e., -Glu^{247}-Lys^{248}-Glu^{249}- to -Gln^{247}-Leu^{248}-Gln^{249}-), the ability of the receptor to activate transducin-mediated GTPase activity is restored (Franke *et al.*, 1988). This finding tempers the simplistic postulate that a electrostatic interaction between the positively charged Lys^{248} and transducin governs rhodopsin–transducin dynamics.

A unique approach to the question of rhodopsin–transducin interactions was taken by Hamm *et al.* (1988). A series of peptides derived from the sequence of the α subunit of transducin were studied for their ability to compete with transducin for binding to rhodopsin, and to induce conformational changes within rhodopsin that mimic those which occur upon interaction with transducin. Two regions near the *C*-terminus of the α subunit of transducin, Glu^{311}–Val^{328} and Ile^{340}–Phe^{350}, were able to block the binding of transducin to rhodopsin and stabilize the activated form of rhodopsin. Further studies showed that a cysteine at position 321 of the first peptide was required for these effects. This strategy has general utility because, by defining the amino acid residues on any given G protein that are responsible for its interaction with a receptor, one may develop a better understanding of the nature of the molecular mechanisms that govern interaction of these proteins.

5. Muscarinic acetylcholine receptors. Several studies have focused on identifying domains within muscarinic acetylcholine receptors that are responsible for G protein interactions. Kubo *et al.* (1988) created receptor chimeras of m_1 and m_2 subtypes, exchanging their respective third intracellular loops. Normally the m_1 subtype, which activates PI turnover, can open a Ca^{2+}-dependent ion channel, whereas the m_2 subtype cannot. Chimeric m_1 receptors possessing the third intracellular loop of the m_2 subtype, however, were unable to stimulate current; conversely, chimeric m_2 receptors possessing the third intracellular loop from the m_1 subtypes were able to mediate this effect. It was concluded that the third intracellular loops of these receptors determine receptor G protein specificity (Kubo *et al.*, 1988). Similar results were obtained by Wess *et al.* (1990) in studies with chimeras of human m_2 and rat m_3 muscarinic acetylcholine receptors. It was noted, however, that the maximal functional responses mediated by the chimeric receptors were quantitatively far lower than those produced by the corresponding native m_2 and m_3 receptors. It was concluded that there may be other domains in addition to the third intracellular loop that are necessary for complete G protein coupling. Alternatively, it is possible that the large stretches of 'foreign' sequences introduced into these receptors take on a conformation that is significantly different from the conformation that would be taken by the native sequence, resulting in receptors which cannot couple in a maximally efficient way (Wess *et al.*, 1990).

To identify more precisely the regions within the third intracellular loop of muscarinic receptors that may confer G protein coupling specificity, other studies focused on a 16–17-amino-acid region at the *N*-terminal end of this loop (i.e. the amino acids of the loop adjacent to the end of transmembrane helix V) (Wess *et al.*, 1989). Analysis of all muscarinic subtypes revealed that the sequence in this segment is highly conserved among m_1, m_3 and m_5 muscarinic acetylcholine receptors, i.e. those that activate phospholipase C, but is distinct from the corresponding segment of the m_2 and m_4 muscarinic acetylcholine receptors, i.e. those that inhibit adenylate cyclase, the latter having their own conserved sequence. An m_2 chimeric receptor containing the 17-amino-acid stretch from the *N*-terminal end of the third intracellular loop of the m_3 receptor behaved like the wild-type m_3 muscarinic acetylcholine receptor, in that it triggered an increase in PI hydrolysis in response to carbachol, albeit with a high EC_{50} value. In addition, the m_3 muscarinic acetylcholine receptor chimera containing the corresponding 16-amino-acid sequence from the m_2 receptor lost its capacity for activating PI hydrolysis (Wess *et al.*, 1989, 1990). Thus it appears that this 16–17-amino-acid sequence at the *N*-terminal end of the third intracellular loops of the m_2 and m_3 muscarinic acetylcholine receptors may play a key role in determining the ability of these receptors to couple to the G protein(s) that activate phospholipase C. However, this region is apparently not the only determinant of coupling selectivity, since the m_2 chimeric receptor which contains the 17-amino-acid stretch from the m_3 receptor retains its ability to inhibit adenylate cyclase, whereas the m_2 chimeric receptor possessing the entire third intracellular loop of the m_3 receptor does not (Wess *et al.*, 1990). It is of note that deletion of up to 123 of the 156 amino acids of the central portion of the third intracellular loop of the mouse m_1 muscarinic acetylcholine receptor can be carried out without decreasing the coupling of the receptor to PI turnover (Shapiro & Nathanson, 1989), supporting the hypothesis that the membrane-proximal sequences of this loop are determinant(s) of G protein specificity.

Different results were obtained by Wong *et al.* (1990), who constructed chimeras in which the second or third intracellular loops, or portions thereof, of the human m_1 muscarinic acetylcholine receptor were substituted with the analogous sequences derived from the turkey β-adrenergic receptor. It was found that virtually the entire third intracellular loop of the m_1 receptor (residues 211–364) could be replaced with the corresponding β-adrenergic sequence without a loss in the ability of the receptor to stimulate PI turnover. Replacement of the second intracellular loop of the m_1 receptor with the β-adrenergic receptor sequence also did not diminish the receptor efficacy for activation of phospholipase C. However, replacement of both the second and third intracellular loops resulted in a 75% reduction in maximal PI turnover; this suggested that these intracellular loops interact in some manner to determine G protein specificity (Wong *et al.*, 1990).

Domains that undergo post-translational modification

1. N-Glycosylation of the *N*-terminus. The majority of G protein-coupled receptors sequenced to date contain one or more consensus sequences for N-glycosylation of asparagine residues [Asn-Xaa-(Ser/Thr)] clustered within the extracellular *N*-terminus (Fig. 3). In fact, it has been noted that the *N*-termini of members of the opsin family have no conserved sequences other than two or more canonical N-glycosylation sites (Applebury & Hargrave, 1986); the same is true for the five muscarinic acetylcholine receptor subtypes (Bonner, 1989).

Glycosylation of the *N*-terminus of several G protein-coupled receptors has been confirmed using biochemical and/or genetic approaches. Hargrave (1977) sequenced a 16-residue trypsin fragment corresponding to the *N*-terminus of bovine rhodopsin which contained two glycosylated asparagine residues, Asn^2 and Asn^{15}. Dohlman *et al.* (1987) showed that a carboxypeptidase Y fragment containing the *N*-terminus of the hamster lung β_2-adrenergic receptor was sensitive to a stepwise digestion with endoglycosidase F, suggesting that at least two potential sites, presumed to be Asn^6 and Asn^{15}, were N-glycosylated. More recently, Rands *et al.* (1990) used site-directed mutagenesis to definitively identify Asn^6 and Asn^{15} as glycosylation sites in the hamster β_2-adrenergic receptor. A series of mutant receptors was

created in which one or both asparagine residues were converted to glutamine, or both potential N-glycosylation sites were removed by deletion mutagenesis (deletion of residues 6–15). Wild-type hamster β_2-adrenergic receptors expressed in COS-7 cells displayed an M_r of 67000 on immunoblots, whereas the [Gln^6, Gln^{15}] and the del-6–15 mutant receptors had an M_r of 43000, which is similar to that of β_2-adrenergic receptors expressed in cells treated with the glycosylation inhibitor tunicamycin or with endoglycosidases. The single-substituted Gln^6 or Gln^{15} β_2-adrenergic receptor mutants displayed intermediate M_r values. Furthermore, it was demonstrated that [^{3}H]glucosamine, which is normally incorporated into the wild-type β_2-adrenergic receptor, was not incorporated into the deletion mutant lacking residues 6–15. Although ligand binding to these mutants was essentially normal, receptor activation of adenylate cyclase was reduced. The latter effect may be related to the fact that the glycosylation-deficient mutant receptors, especially the [Gln^6, Gln^{15}]β_2-adrenergic receptor, displayed an altered subcellular distribution, with a significant reduction (up to 50%) in the amount of receptor appearing at the cell surface. These data suggest that N-glycosylation may play a critical role in directing the β_2 receptor to its proper cellular location (Rands *et al.*, 1990).

In a parallel study, van Koppen & Nathanson (1990) substituted the three asparagine residues within potential N-glycosylation sites (positions 2, 3 and 6) of the *N*-terminus of the porcine m_2 muscarinic acetylcholine receptor with Asp or Glu, in order to evaluate the role of glycosylation in the function of this receptor. The triple-mutant receptors were not glycosylated; however, receptor targeting to the cell membrane, ligand binding affinities and ability to inhibit adenylate cyclase activity were not generally different from those of wild-type m_2 muscarinic acetylcholine receptors.

There are examples of G protein-linked receptors that lack consensus sequences for N-linked glycosylation, in either their *N*-termini or their extracellular loops. For example, both human (Weinshank *et al.*, 1990) and rat (Zeng *et al.*, 1990) α_{2B}-adrenergic receptors, which lack N-glycosylation consensus sequences, have been expressed in mammalian cells and display the expected pharmacological properties. Also, the RDC8 clone (Libert *et al.*, 1989), identified as an adenosine A_2 receptor (Maenhaut *et al.*, 1990), lacks glycosylation sequences. These studies suggest that N-linked glycosylation is not absolutely necessary for the correct targeting of G protein-linked receptors to the cell membrane in all cases. Whether there is any other functional significance to the lack of glycosylation in this subset of G protein-linked receptors remains to be determined.

2. Receptor phosphorylation and desensitization. Desensitization is generally defined as the attenuation of a biological response upon prolonged exposure to agonists. Distinctly different but potentially interrelated desensitization phenomena have been described, based primarily on studies on the β-adrenergic receptor-coupled adenylate cyclase system (for a review, see Sibley & Lefkowitz, 1985). The first phenomenon involves an agonist-induced functional uncoupling of the receptor from its G protein. In homologous desensitization, prolonged agonist exposure results in an uncoupling of the agonist-occupied receptor from its G protein and effector system; the responsiveness of other receptors utilizing the same G protein/effector system are not affected. In heterologous desensitization, prolonged agonist exposure results not only in the attenuation of the response of the occupied receptor, but also in that of other receptors which are coupled to the same G protein/effector system.

Other desensitization phenomena involve a decrease in the number of receptors at the cell surface. Sibley *et al.* (1986) have shown that agonist exposure triggers the sequestration of β-adrenergic receptors into subcellular membrane vesicles within a relatively short time span (minutes). The sequestered receptors can be returned to the plasma membrane following removal of agonist, thereby restoring the full complement of membrane receptors (Sibley *et al.*, 1986). A related event, usually occurring on a longer time scale (minutes to hours), is down-regulation, in which the total number of receptors is decreased. In this case, the reappearance of receptors at the cell surface requires new protein synthesis. Below are reviewed some of the molecular events associated with desensitization, and the evidence implicating certain domains of G protein-linked receptors in these processes.

(*a*) Rhodopsin. It has been known since the early 1970s that rhodopsin undergoes a light-dependent, ATP-dependent phosphorylation (Kuhn & Dreyer, 1972; Bownds *et al.*, 1972; Frank *et al.*, 1973). The enzyme responsible for this activity, rhodopsin kinase, utilizes only the photoactivated form of rhodopsin as a substrate (Weller *et al.*, 1975; Frank & Buzney, 1975; Kuhn, 1978; Shichi & Somers, 1978). Protein mapping studies indicated that rhodopsin is phosphorylated at multiple serine and threonine sites clustered in the *C*-terminus (Hargrave *et al.*, 1980; Barclay & Findlay, 1984; Thompson & Findlay, 1984); up to 9 mol of phosphate have been reported per mol of pigment (Wilden & Kuhn, 1982). Phosphorylation of rhodopsin has been proposed to have a signal-terminating function; this was suggested when it was found that addition of ATP to rod outer segments attenuated the activation of cyclic GMP phosphodiesterase in this system (Liebman & Pugh, 1980). Furthermore, Miller & Dratz (1984) demonstrated that removal of most of the phosphorylation sites on rhodopsin by proteolytic cleavage of the *C*-terminus prevented the ATP-dependent deactivation of cyclic GMP phosphodiesterase.

It was subsequently discovered that a 48000 Da protein intrinsic to rod outer segments is capable of binding to phosphorylated, photoactivated rhodopsin. This protein, known as arrestin, mediates the quenching of phototransduction (Kuhn *et al.*, 1984). Arrestin binding to phosphorylated rhodopsin acts to block the binding and activation of transducin, and can almost completely suppress the ability of the receptor to activate phosphodiesterase (Wilden *et al.*, 1986). It is thought that the sites on rhodopsin for binding of transducin and arrestin may overlap (Wilden *et al.*, 1986). Arrestin binding to rhodopsin also blocks the additional phosphorylation of rhodopsin by rhodopsin kinase (Buczylko *et al.*, 1991) and interferes with the dephosphorylation of rhodopsin by protein phosphatase 2A (Palczewski *et al.*, 1989). The net effect of these actions is to facilitate the continued association between phosphorylated rhodopsin and arrestin. Regeneration of the functional phototransduction pathway is dependent on the slow release of arrestin from rhodopsin.

(*b*) β-Adrenergic receptors. Certain elements of β-adrenergic receptor desensitization are analogous to those of the rhodopsin system. Initial studies on β-adrenergic receptor desensitization indicated that the receptors undergo phosphorylation as a result of prolonged exposure to agonists (Stadel *et al.*, 1983; Sibley *et al.*, 1985). In a series of studies, Benovic *et al.* (1986, 1987*b*) characterized one of the enzymes responsible for this phosphorylation, a cyclic AMP-independent cytosolic 80000 Da protein designated β-adrenergic receptor kinase. An early event in this process is the translocation of β-adrenergic receptor kinase from the cytosol to the plasma membrane (Strasser *et al.*, 1986). In a manner akin to rhodopsin kinase, which phosphorylates only the photoactivated form of rhodopsin, β-adrenergic receptor kinase was shown to phosphorylate multiple serine and threonine residues (up to 9 mol of phosphate/mol of receptor) on only the agonist-occupied form of the receptor (Benovic *et al.*, 1986). Removal of the *C*-terminus of reconstituted hamster lung β_2-adrenergic receptors by carboxypeptidase Y treatment removed most, but not all, of the phosphorylation sites, suggesting that

this serine-threonine-rich region is the major site of receptor regulation by phosphorylation (Dohlman *et al.*, 1987). The similarity of the two systems is further demonstrated by the fact that in cell-free systems β-adrenergic receptor kinase can utilize rhodopsin as a substrate (Mayor *et al.*, 1987).

The β_2-adrenergic receptor is also a substrate of cyclic AMP-dependent protein kinase A, suggesting a feedback mechanism of receptor desensitization based on activation of adenylate cyclase (Bouvier *et al.*, 1987). There are two sites on the β_2-adrenergic receptor, at positions 259–262 on the third cytoplasmic loop and positions 343–348 on the *C*-terminus, which contain the Lys/Arg-Arg-X-(Xaa)-Ser- consensus sequence that is thought to serve as a site for protein kinase A phosphorylation (Dixon *et al.*, 1986). *In vitro* mutagenesis has been used to attempt to establish a relationship between phosphorylation of the β-adrenergic receptor and various facets of the desensitization process. Results have not always been clear cut, since desensitization appears to be a pleiotropic response, and different cell systems may utilize different combinations of desensitizing mechanisms. Nevertheless, some trends are emerging.

Several investigations have addressed the question of whether or not phosphorylation of β-adrenergic receptors results in the functional uncoupling of the receptor from G_s. Seminal studies by Sibley *et al.* (1986) showed that phosphorylated β-adrenergic receptors, purified from desensitized frog erythrocytes and reconstituted into G_s-containing phospholipid vesicles, activated G_s (as measured by GTPase activity) less efficiently than did non-phosphorylated controls. Similar results were obtained when purified β_2-adrenergic receptor was phosphorylated *in vitro* with either protein kinase A or β-adrenergic receptor kinase (Benovic *et al.*, 1987*a*,*b*).

To test whether phosphorylation of the protein kinase A consensus sites and/or the β-adrenergic receptor kinase sites (i.e. the serines and threonines of the *C*-terminus) results in an uncoupling of the β_2-adrenergic receptor, a series of mutations altering these putative phosphorylation domains were created; the ability of these mutant receptors to undergo desensitization was then studied (Bouvier *et al.*, 1988; Hausdorff *et al.*, 1989). Phosphorylation of different domains of the human β_2-adrenergic receptor was associated with different densensitization phenomena (Hausdorff *et al.*, 1989). CHW cells expressing human β_2-adrenergic receptors that were pre-exposed to relatively low (20 nM) concentrations of isoprenaline display a shift to the right in the dose–response curve for activation of adenylate cyclase, as compared with untreated controls. Since there is only minor amount of receptor sequestration under these conditions, this shift apparently reflects a functional uncoupling of the receptor from G_s. Importantly, this rightward shift is greatly attenuated in cells expressing a mutant β_2-adrenergic receptor in which the serine residues of the protein kinase A phosphorylation sites are replaced by alanine. There was no attenuation of this shift in cells expressing a second mutant receptor in which the serine and theonine residues of the *C*-terminus that are believed to serve as phosphorylation sites for β-adrenergic receptor kinase had been converted to alanine or glycine. This suggested that receptor phosphorylation at the protein kinase A sites was responsible for receptor uncoupling induced by exposure to low levels of agonist (Hausdorff *et al.*, 1989).

Upon exposure of cells expressing wild-type receptors to high concentrations of isoprenaline (2 μM), the desensitization mechanism is altered; there is not only a rightward shift in the dose–response curve for isoprenaline-mediated activation of adenylate cyclase, but also a decrease in the efficacy of this response. Significantly, the receptor mutants missing either the phosphorylation sites for protein kinase A or β-adrenergic receptor kinase did not undergo a loss of efficacy for adenylate cyclase stimulation. Presumably, receptor phosphorylation at both of these domains is needed to effect this loss of efficacy.

Other studies with this system have demonstrated that the longer cells expressing wild-type human β_2-adrenergic receptors are preincubated with high concentrations of isoprenaline (2 μM), the greater the observed loss of efficacy for activating adenylate cyclase, up to a maximum of 180 min (Bouvier *et al.*, 1988). Two mutants of the human β_2-adrenergic receptor that lack β-adrenergic receptor kinase phosphorylation sites, one truncated after amino acid 365 (i.e. missing the serine/threonine-rich portion of the *C*-terminus), and one in which 11 serine and threonine residues within the *C*-terminus were substituted with either alanine or glycine, were examined for their ability to undergo desensitization under these same conditions. Both displayed a short-term (i.e. within 15 min after addition of agonist) delay in the onset of desensitization. However, after 180 min of exposure to isoprenaline, both mutant receptors were maximally desensitized. In this system, early desensitization events were agonist-specific, i.e. homologous desensitization, with other desensitization mechanisms (e.g. heterologous) being activated at longer agonist exposure times. These data implied that β-adrenergic receptor kinase phosphorylation is, at least in part, responsible for early-onset homologous desensitization, but has little or no role in the later events (Bouvier *et al.*, 1988).

Recent evidence has also linked receptor phosphorylation with heterologous desensitization of the β_2-adrenergic receptor. A system has been described in which the hamster β_2-adrenergic receptor expressed in L cells undergoes heterologous desensitization in response to short (< 10 min) exposure to relatively low (10–50 nM) concentrations of adrenaline or of another activator of adenylate cyclase, prostaglandin E_1 (PGE_1); this heterologous desensitization is characterized by a 2–3-fold increase in the $K_{act.}$ of adrenaline for adenylate cyclase stimulation without receptor sequestration, or a loss of maximal efficacy for adenylate cyclase activation (Clark *et al.*, 1989). In cells expressing a deletion mutant lacking residues corresponding to the consensus site for protein kinase A phosphorylation on the third cytoplasmic loop (i.e. residues 259–262), exposure to either adrenaline or PGE_1 did not produce desensitization. Cells expressing a β_2-adrenergic receptor deletion mutant that lacks the other consensus site for protein kinase A phosphorylation, i.e. residues 343–348 in the *C*-terminus, or one in which the serine-rich *C*-terminus has been removed by a truncation mutation at residue 354, displayed normal heterologous desensitization in response to prior adrenaline or PGE_1 exposure. These findings indicate that phosphorylation of the consensus site at residues 259–262 on the third cytoplasmic loop by protein kinase A is necessary for heterologous desensitization in this system (Clark *et al.*, 1989).

On the other hand, it is apparent that agonist-induced sequestration of the β_2-adrenergic receptor can occur in mutant receptors that lack most of the major putative phosphorylation target sites. For example, Strader *et al.* (1987*c*) constructed a hamster β_2-adrenergic receptor mutant in which both protein kinase A phosphorylation sites were deleted and the *C*-terminus was truncated after residue 395; this receptor displayed normal isoprenaline-induced sequestration. Similarly, Hausdorff *et al.* (1989) constructed human β_2-adrenergic receptor mutants in which both the serines of the protein kinase A consensus sites and the serines and threonines of the *C*-terminus were replaced with alanine or glycine: these mutant receptors displayed greatly decreased agonist-stimulated receptor phosphorylation, but normal sequestration when expressed in CHW cells undergoing prolonged exposure to isoprenaline. Thus a link between receptor phosphorylation and receptor sequestration has yet to be definitively established.

As an alternative to the phosphorylation hypothesis, it has been suggested that the regions of the β_2-adrenergic receptor that appear to be necessary for activation of G_s are also necessary for receptor sequestration. A series of hamster β_2-adrenergic receptors with deletions in the third cytoplasmic loop were studied for their ability to activate G_s and adenylate cyclase and to undergo sequestration. The only two mutant receptors that did not undergo sequestration in desensitization studies on transfected L cells, del-239–272 and del-222–229, were completely unable to activate G_s or adenylate cyclase (see earlier) (Strader *et al.*, 1987*c*; Cheung *et al.*, 1989). Deletion mutants such as del-230–262 that could partially stimulate adenylate cyclase underwent significant, although less than maximal, sequestration. Based on these findings, it was postulated that the same structural features of the receptor responsible for G protein coupling are also involved in the sequestration process. However, in a subsequent study, these investigators found that coupling to G_s was not required for sequestration of β_2-adrenergic receptors. Replacement of residues 222–229 of the hamster β_2-adrenergic receptor with the analogous region of the human m_1 muscarinic acetylcholine receptor yielded a hybrid receptor that was unable to trigger G_s activation (as measured by an inability to cause a rightward shift in isoprenaline competitive binding curves in the presence of guanosine 5′-[β,γ-imido]triphosphate) or stimulate adenylate cyclase; nevertheless, this altered receptor underwent nearly normal isoprenaline-mediated sequestration (Cheung *et al.*, 1990). Thus at the present time the structure(s) within the β-adrenergic receptor which are responsible for sequestration have not been defined.

Finally, there is some evidence that agonist-induced receptor phosphorylation may play a limited role in the down-regulation of β-adrenergic receptors. Bouvier *et al.* (1989) discovered that when CHW cells expressing wild-type human β_2-adrenergic receptors are treated with dibutyryl cyclic AMP there is a gradual loss of total receptor number over the course of 24 h. Only a negligible amount of receptor sequestration is induced by this treatment. The onset of receptor down-regulation is delayed, however, in cells expressing mutant β_2-adrenergic receptors whose protein kinase A consensus sites were disrupted by insertion or substitution. It was suggested that phosphorylation of the receptor might increase the rate of down-regulation, at least in the initial few hours after dibutyryl cyclic AMP exposure, perhaps by enhancing the susceptibility of the receptor to proteases. However, the major mechanism by which dibutyryl cyclic AMP causes the long-term down-regulation of the wild-type receptor is by inducing a decrease in the steady-state levels of β_2-adrenergic receptor mRNA; this is not altered in cells expressing the mutant receptor. It therefore appears that, at least over the long term, the process of cyclic AMP-mediated down-regulation occurs independently of receptor phosphorylation (Bouvier *et al*, 1989).

In all likelihood, other mechanisms ancillary to or independent of receptor phosphorylation may play a role in agonist-induced desensitization. It was shown that the uncoupling of the β_2-adrenergic receptor from G_s in a reconstituted phospholipid vesicle induced by treatment with β-adrenergic receptor kinase could be enhanced by the addition of the retinal-derived protein, arrestin (Benovic *et al.*, 1987*a*). Recently, a cDNA clone encoding a protein homologous to arrestin was isolated. This protein, termed β-arrestin, was found to inhibit the activity of phosphorylated β-adrenergic receptors by more than 75%, but to not affect that of rhodopsin (Lohse *et al.*, 1990). These findings suggest that β-adrenergic receptor kinase and β-arrestin may function in concert to mediate homologous desensitization of β-adrenergic receptors (Lohse *et al.*, 1990).

Other domains and/or amino acid residues may also influence receptor down-regulation. Valiquette *et al.* (1990) have obtained evidence that two tyrosine residues in the *C*-terminus of the human β_2-adrenergic receptor, Tyr^{350} and Tyr^{354}, play some role in the ability of this receptor to undergo down-regulation. These investigators found that conversion of both of these residues to alanine produces a mutant β_2-adrenergic receptor that is less susceptible to the effects of prolonged (up to 24 h) isoprenaline exposure relative to wild-type receptors when expressed in CHW cells. These mutations do not prevent the receptor from undergoing normal sequestration, however. It was speculated that these tyrosine residues may be important for interactions with proteins involved in endocytosis, such as those involved in the formation of clathrin-coated vesicles. This is based on studies with non-G-protein-linked membrane receptors, such as the mannose 6-phosphate receptor, whose *C*-terminal tyrosines appear to be necessary for agonist-induced endocytosis involving clathrin-coated vesicles (Lobel *et al.*, 1989). The role of clathrin-coated vesicles in the down-regulation of G protein-linked receptors has yet to be thoroughly examined.

PROSPECTUS

What are the archetypal features shared by all G protein-coupled receptors? In the 9 years since the report of the molecular cloning of the first G protein receptor, more than 100 distinct receptor subtypes of this large gene family have been sequenced, permitting us to identify and begin to examine the role of common structural motifs. In this review we have catalogued some of the commonalities among these receptors. It is debatable whether any of these features other than the presence of seven transmembrane domains constitute a *sine qua non* for G protein-coupled receptors: exceptions have already been found for many of these motifs. Such exceptions are not surprising, given the large number of members of this gene family and the diversity of physiological functions that these receptors modulate. Nevertheless, collectively these features establish a kinship between these specialized proteins and point to a common ancestry. Ultimately, one of the major goals of structure–function studies of receptors is to identify the features that distinguish one receptor subtype from another. *In vitro* mutagenesis will likely serve as an important tool in this process. Understanding receptor diversity at the molecular level constitutes a major goal of receptor research, as it would provide the framework for the design of subtype-specific pharmacological agents.

Since this review was originally submitted, the cloning and sequence analysis of the secretin (Ishihara *et al.*, 1991), parathyroid hormone (Juppner *et al.*, 1991) and calcitonin (Lin *et al.*, 1991) receptors has suggested the existence of a new subfamily of G protein-linked receptors. This family of receptors contains seven potential transmembrane domains, but shows no sequence similarity with other reported G protein-coupled receptors.

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