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Dimerization and Domain Swapping in G-Protein-Coupled Receptors: A Computational Study

Paul R. Gouldson, Ph.D., Christopher Higgs, Ph.D., Richard E. Smith, B.Sc., Mark K. Dean, B.Sc., George V. Gkoutos, M.Sc., and Christopher A. Reynolds, Ph.D.

In recent years there has been an increasing number of reports describing G protein-coupled receptor (GPCR) dimerization and heterodimerization. However, the evidence on the nature of the dimers and their role in GPCR activation is inconclusive. Consequently, we present here a review of our computational studies on G protein-coupled receptor dimerization and domain swapping. The studies described include molecular dynamics simulations on receptor monomers and dimers in the absence of ligand, in the presence of an agonist, and in the presence of an antagonist (or more precisely an inverse agonist). Two distinct sequence-based approaches to studying protein interfaces are also described, namely correlated mutation analysis and evolutionary trace analysis. All three approaches concur in supporting the proposal that the dimerization interface includes transmembrane helices 5 and 6. These studies cannot distinguish between domain swapped dimers and contact dimers as the models used were restricted to the helical part of the receptor. However, it is proposed that for the purpose of signalling, the domain swapped dimer and the corresponding contact dimer are equivalent. The evolutionary trace analysis suggests that every GPCR family and subfamily (for which sufficient sequence data is available) has the potential to dimerize through this common functional site on helices 5 and 6. The evolutionary trace results on the G protein are briefly described and these are consistent with GPCR dimerization. In addition to the functional site on helices 5 and 6, the evolutionary trace analysis identified a second functional site on helices 2 and 3. Possible roles for this site are suggested, including oligomerization.
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G protein-coupled receptors (GPCRs) are integral membrane proteins involved in cell signalling (Strader et al. 1994; Wess 1998). The basic aspects of GPCR activation, as presented in contemporary undergraduate textbooks and shown schematically in Figure 1, have been known for a while but the revolutionary concept of GPCR dimerization has challenged this conventional wisdom (Bockaert and Pin 1999; Gouldson et al. 1998; Hebert and Bouvier 1998).

In the last few years, the evidence for GPCR dimers has grown substantially and so the observation that GPCR dimerize and heterodimerize can no longer be

From the Department of Biological Sciences, Central Campus, Wivenhoe Park, Colchester, Essex, United Kingdom.

Address correspondence to: Dr. Christopher A. Reynolds, Department of Biological Sciences, Central Campus, Wivenhoe Park, Colchester, Essex, CO4 3SQ, United Kingdom.

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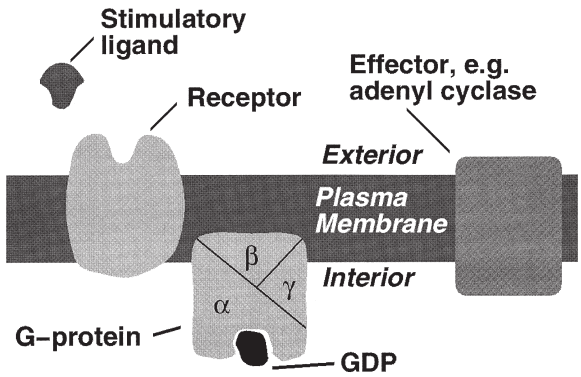

Figure 1. A schematic diagram showing the interaction of G protein-coupled receptors (GPCRs) with their ligands and G protein. The diagram is typical of those shown in contemporary molecular cell biology textbooks.

refuted (Bai et al. 1998; Ciruela et al. 1995; Cvejic and Devi 1997; Hebert et al. 1996; Jones et al. 1998; Jordan and Devi 1999; Kaupmann et al. 1998; Kolakowski 2000; Maggio et al. 1993a, 1996; Monnot et al. 1996; Ng et al. 1996; Rocheville et al. 2000; Romano et al. 1996; White et al. 1998; Xie et al. 1999). Moreover, many observations related to GPCR function are hard to explain by any unified theory that does not involve dimerization (Gouldson et al. 1998). Nevertheless, definitive proof that GPCRs are involved in the normal activation and or deactivation mechanisms of GPCRs is elusive. Against this background, we have carried out both sequence studies and computer simulations aimed at understanding the dimerization process in general, and understanding whether this dimerization process could also involve domain swapping. Here, we review our research to date and present some novel research findings from sequence studies on G protein-coupled receptors.

The GPCR super-family members are characterized by a heptahelical fold and intracellular loops that are involved in G protein coupling (Schöneberg et al. 1999; Strader et al. 1994). The super-family is split into families and subfamilies according to function and specificity to their endogenous agonist (Watson and Arkinstall 1994). In some families, such as the catecholamines, the endogenous agonist binds within the transmembrane region. In the class A peptide family, the peptide ligand also binds to extracellular regions of the receptor (N terminus and extracellular loops). In other families, such as $GABA_B$, a class C receptor, the ligand binds wholly within the N-terminal region. Sequence similarity between the super-families is low, but within the families, it is usually over the 35% threshold that allows automatic multiple sequence alignment to be used with confidence. Nevertheless, there are many similarities shared by the GPCRs and so the general GPCR activation process shown schematically in Figure 1 probably applies to all families.

It is accepted that a ligand, typically a hormone or a neurotransmitter, binds to the receptor. The ligand-receptor complex then interacts with a heterotrimeric G protein, such that the GDP, is released from the G protein and replaced by GTP. On binding GTP, the G protein dissociates into G_α and $G_{\beta\gamma}$ subunits, each of which has its own role in signal transduction—in Figure 1, the next stage in the activation process is an interaction between G_α and adenylate cyclase that continues until the G protein has hydrolysed the GTP back to GDP.

It has been interesting to observe the change in the relative magnitudes of the receptor and G protein in such figures over the last 10 years or so; the key scientific question we are seeking to address here is whether there will be similar changes in the stoichiometries and relative orientations. Before presenting our contribution to this debate on the nature and possible role of the dimer it is necessary to introduce the idea of domains in GPCRs.

DOMAINS IN G-PROTEIN-COUPLED RECEPTORS

Autonomous Folding Units

A number of authors use the phrase domain to refer to a small unit such as a transmembrane helix, but it is more usual to use the phrase domain to refer to a pseudo-independent structural unit. Indeed, several experiments have illustrated the ability of GPCR receptor fragments to function as autonomous folding units and these are summarised in Figure 2. This figure shows that it is possible to split GPCRs into fragments that can be co-expressed, and depending where the receptor is

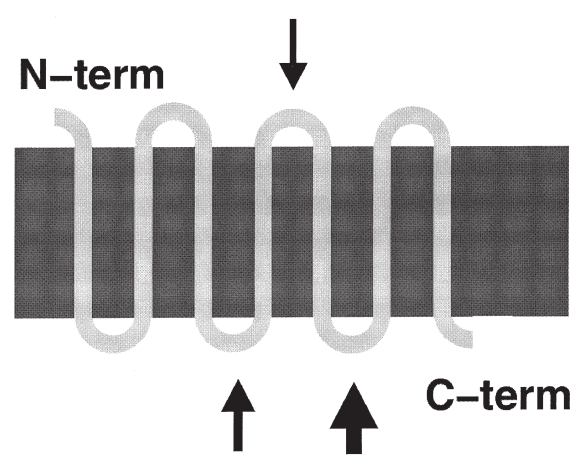

Figure 2. Domains in G protein-coupled receptors (GPCRs), as identified by experiments involving the co-expression of GPCR fragments. Typically, binding may be observed if the receptor is cut in extracellular or intracellular loop 2 but binding and activity is usually only observed is the receptor is cut in intracellular loop 3.

split, it is possible for the co-expressed fragments to show some or all of the properties of the wild type receptor. While the individual fragments are non-functional, some activity can be regained if the receptor is split between helices 3 and 4 or between helices 4 and 5. However, full activity on co-expression, namely binding and G protein activation, has only be observed when the receptor is split between helices 5 and 6, as shown for the rhodopsin (Ridge et al. 1996), adrenergic (Kobilka et al. 1988), muscarinic (Schöneberg et al. 1995), vasopressin (Schöneberg et al. 1996), GNRH (Gudermann et al. 1997), and the neurokinin receptor (Nielsen et al. 1998). Thus, the N-terminal and helices 1–5 constitute the A domain while helices 6 and 7 through to the C-terminal constitute the B domain.

Docking Studies

The idea that GPCRs constitute separate domains has been applied to the problem of docking adrenergic ligands into a model of the β_2-adrenergic receptor. The GPCR heptahelical bundle is a compact structure and so there is little free space to use in docking of adrenergic ligands using interactive molecular graphics. One solution to this problem involves a 3-stage docking process (Gouldson et al. 1997a). In the first stage, the B domain is moved away from the A domain as shown in Figure 3a.

In the second stage, the ligand is docked against the A domain using just 2 anchor points: (i) the key aspartic acid residue on helix 3—which binds the charged amine group common to adrenergic ligands; and (ii) one of the two hydrophobic regions either side of helix 3 lying between helices 1 and 3 or between helices 3 and 5—which binds the hydrophobic aromatic region. This process is illustrated in Figure 3b. (Typical adrenergic ligands are shown in Figure 4.)

In the third stage, the B domain is allowed to move back to the A domain during the course of a molecular dynamics simulation (Grant and Richards 1995; Karplus and Petsko 1990) to generate the final docked conformation (Figure 3c). There are two advantages of this process. Firstly, the process creates additional room, enabling the system to undergo enhanced phase space sampling and thus give the drug additional opportunities to find the lowest energy conformation. Secondly, the process may mimic the way the docking process occurs in nature. Molecular modelling shows that the N-terminus and the three extracellular loops create a tight canopy over the receptor and it is not clear how a ligand can bind unless there is substantial breathing of both the extracellular loops and the helical domains. Indeed, Brownian dynamics simulations of the extracellular loops of the adrenergic receptor have shown that movement of the loops alone may not be sufficient to permit binding of all but the smallest ligands (Kamiya and Reynolds 1999). Thus, the domain movement exploited in this docking protocol may even mimic aspects of the domain swapping process (Gouldson et al. 1997a)—see below.

Generally, the receptor-ligand interactions identified through these studies were in broad agreement with other modelling studies on the β_2-adrenergic receptor (Gouldson et al. 1997a). However, two novel observations also came out of these studies, namely the hydrogen bonding interaction between Asn^{312} on helix 7 with propranolol (Suryanarayana et al. 1991; Suryanarayana and Kobilka 1993) and the interaction between Leu^{311} and the amino or methyl amino group of norepinephrine and epinephrine, respectively (see Figure 4). Both of these residues are correlated and in particular they are correlated with receptor subtype as residue 312 is a Leu in the α-adrenergic receptors and an

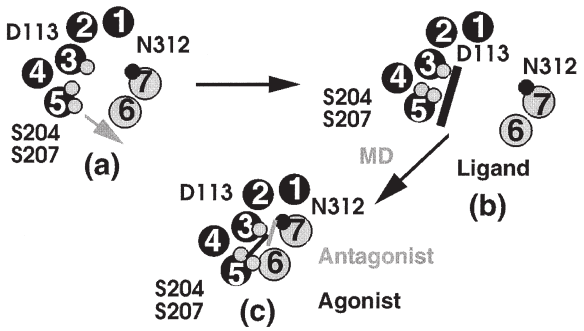

Figure 3. The docking protocol that exploits the domain structure of G protein-coupled receptors (GPCRs) (Gouldson et al. 1997a). The only interactions engineered into this process were between the protonated nitrogen of the drug and the Asp^{113} on helix 3 and between the aromatic ring and the hydrophobic patches. The hydrogen bond interactions to S204 and S207 (agonist) or N312 (antagonist) on helices 5 and 7, respectively, formed automatically during the course of the molecular dynamics simulation.

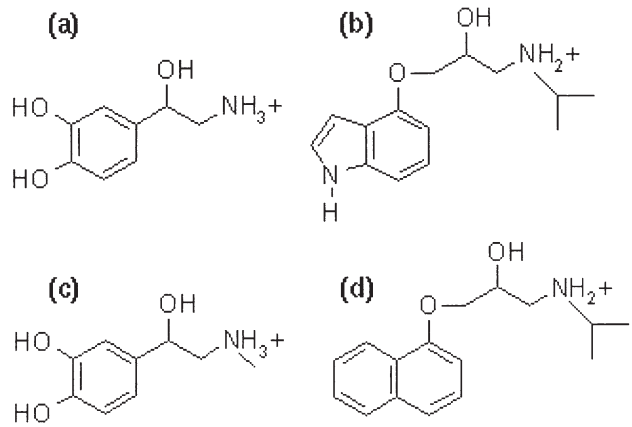

Figure 4. Typical adrenergic ligands, as used in the molecular modelling studies: (a) norepinephrine, (b) pindolol, (c) epinephrine, (d) propranolol.

Asn in the β-adrenergic receptors while residue 311 is a Phe in the β_1 and β_3 and a Leu in the β_2-adrenergic receptors. Correlated residues are explained in Figure 5. Thus, these docking studies offer an explanation for the observation that antagonists containing an ether oxygen (or similarly placed OH group) prefer β receptors over α receptors and the observation that norepinephrine binds preferably to β_1- and β_3-adrenergic receptors while epinephrine binds preferably to β_2-adrenergic receptors. The latter observation has been partially supported by high-level calculations on model systems (Ferenczy et al. 1997). The docking studies also confirm the usefulness of correlated mutation analysis (Gobel et al. 1994; Pazos et al. 1997; Singer et al. 1995) in studying GPCRs and we will return to this later after we have introduced the idea of domain swapping.

Molecular Dynamics Simulations on GPCR Activation

Molecular dynamics simulations on GPCRs in the absence of ligand and in the presence of antagonist, partial agonist, and full agonist enable us to study the changes in GPCR structure in response to the individual atomic forces arising between the ligand and the receptors. There is considerable debate as to whether such simulations are justified because there is no high resolution X-ray crystal structure of a GPCR but rather simulations have to be based on homology models, which are generally not noted for their reliability. However, for GPCRs the homology models are based on a series of low resolution cryoelectron microscopy structures (Baldwin et al. 1997; Schertler 1998; Unger et al. 1997) and the homology modelling process is assisted by an enormous wealth of sequence (Horn et al. 1998; Vriend 2000) mutation (Kristiansen et al. 1996, 2000) and biophysical data (Gouldson et al. 1997a) and so for GPCRs, homology modelling is particularly justified. Indeed, evidence that these models produce useful information comes from the simulations on the activation process—for recent reviews on modelling GPCRs see Flower (1999) and Higgs and Reynolds (2000).

Simulations show that there are essentially no differences in structure between the β_2-adrenergic receptor in the absence of ligand and in the presence of the antagonist propranolol. However, the results from our simulations on the β_2-adrenergic receptor in the presence of the agonist norepinephrine (Figure 6) show that the extracellular region of the receptor is largely unaffected but large changes in the position of helices 5 and 6 are visible at the intracellular side of the receptor (Gouldson et al. 1997a). This is encouraging because this is precisely the region where conformational changes on activation have been detected in other computational (Luo et al. 1994; Scheer et al. 1996; Zhang and Weinstein 1993) and experimental studies (Altenbach et al. 1996; Farahbakhsh et al. 1995; Farrens et al. 1996; Gether et al. 1995, 1997a, 1997b; Javitch et al. 1997).

The studies reveal that conformational changes have also been observed in helix 3 but that is not apparent in Figure 6 because helix 3 of the *apo* and ligand-bound receptors were superimposed. At a simple level, this result is in full accord with our knowledge of GPCR activation since it is generally understood that of the three intracellular loops, intracellular loop three plays the greatest role in receptor activation (Strader et al. 1994; Wess 1998) and a change in helices 5 and 6 would naturally cause a change in the intracellular loop connecting them. However, there are many aspects of GPCR function that cannot be explained solely by conformational changes in the monomer and so we need to consider similar studies on the receptor dimer. First, however, it is beneficial to introduce the concept of domain swapping.

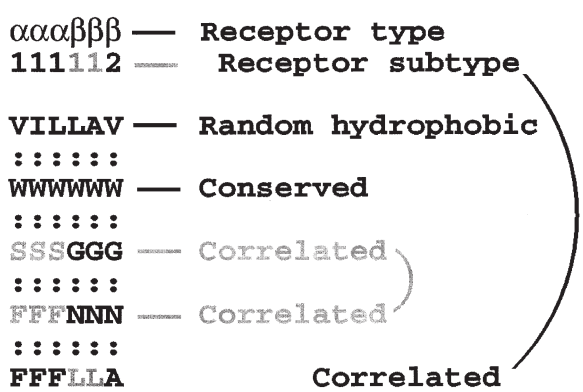

Figure 5. Correlated mutation analysis. The figure shows sequence data for five positions (which are not necessarily sequential) of six adrenergic receptors (containing both α and β types, which themselves may be subdivided into further subtypes). The first position given contains a random hydrophobic residue; the second contains a conserved Trp residue. The third and fourth positions are correlated in that whenever a Ser mutates to a Gly (at position 3) there is a corresponding mutation of Phe to Asn (at position four). The amino acids at the final position are correlated with the receptor subtype.

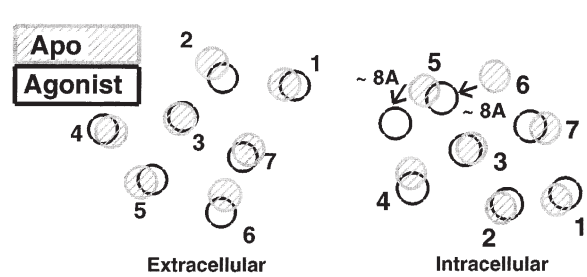

Figure 6. Changes in GPCR helical structure in response to an agonist, as determined during the course of a molecular dynamics simulation.

Domain Swapping

There has been much discussion of dimerization in GPCRs but relatively little discussion on the nature of the dimers. Two basic modes of dimerization are possible for multi-domain proteins, as shown in Figure 7. A monomer is shown in Figure 7a; if two monomers merely touch each other, a contact dimer forms, as shown in Figure 7b, but if the hinge loop opens out the domains can exchange, to form a domain swapped dimer, as shown in Figure 7c.

Domain swapping has been reviewed extensively elsewhere (Bennett et al. 1994, 1995; Murray et al. 1995; Schlunegger et al. 1997; Tegoni et al. 1996). Domain swapped dimers are less common than contact dimers but have the major advantage that the interactions between the domains already present in the monomers can be re-used to form the dimers—thus domain swapping is an efficient way of forming dimerization interfaces. The length of the hinge loop is important in this process and for GPCRs, we should note that intracellular loop three, the hinge loop connecting the two domains, is frequently the longest loop in GPCRs. Moreover, Maggio and coworkers showed that shortening the hinge loop resulted in loss of activity (Maggio et al. 1996).

EVIDENCE FOR G PROTEIN-COUPLED RECEPTOR DIMERIZATION AND THE NATURE OF THE DIMERIZATION INTERFACE

We do not propose to present all of the evidence for GPCR dimers because the evidence has been reviewed elsewhere in the literature (Gouldson et al. 1998; Hebert and Bouvier 1998) and on our web site (Reynolds 2000).

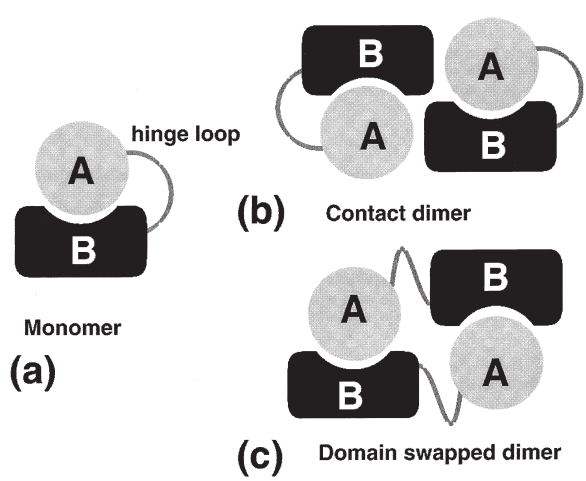

Figure 7. Different modes of dimerization of a two domain protein: **(a)** a two-domain monomer, **(b)** a contact dimer, **(c)** a domain swapped dimer.

However, it is worth noting that evidence for dimerization has now been recorded for the adrenergic, vasopressin (Hebert et al. 1996), angiotensin (Monnot et al. 1996), dopamine (Ng et al. 1996; Zawarynski et al. 1998), muscarinic (Zeng and Wess 1999), adenosine (Ciruela et al. 1995), opioid (Cvejic and Devi 1997; Jordan and Devi 1999), serotonin (Xie et al. 1999), and somatostatin (Rocheville et al. 2000) receptors, a class B GPCR (Kolakowski 2000), and for several class C GPCRs (Bai et al. 1998; Jones et al. 1998; Kaupmann et al. 1998; Romano et al. 1996; White et al. 1998) and so it would be interesting to determine whether all GPCR have the potential to dimerize.

One of the most recent developments is the observation of GPCR heterodimerization. Here, perhaps the most notable development is the observation that the $GABA_B$ receptor subtypes $GABA_BR1$ and $GABA_BR2$ were not functional in isolation but were functional when co-expressed (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998). Significantly, it was also noted that novel pharmacology was observed for ligands acting at the κ and δ opioid receptor heterodimer (Jordan and Devi 1999). Heterodimerization has also been observed for muscarinic subtypes (Maggio et al. 1999; Sawyer and Ehlert 1999), serotonin subtypes (Xie et al. 1999), and for somatostatin subtypes (Rocheville et al. 2000). These references give little information on the location of the dimerization interface. The evidence we have is that disulphide bonds between the receptor canopy may be involved for the calcium sensing and metabotropic glutamate receptors (Bai et al. 1998; Romano et al. 1996) and even for the muscarinic receptors (Zeng and Wess 1999), that the $GABA_B$ heterodimer may be held together via coiled-coils formed between the two C-termini (White et al. 1998), and that helix 6 may be involved in the adrenergic dimerization interface, since a peptide based on helix 6 can inhibit both dimerization and activation (Hebert et al. 1996).

The most illuminating evidence however comes from the studies of Maggio and coworkers on chimeric adrenergic-muscarinic receptors (Maggio et al. 1993a, 1996). The α_2-M3 chimeras, shown schematically in Figure 8a, were naturally inactive—they did not bind ligand and they did not activate the G protein; the complementary M3-α_2 chimera was equally inactive. However, near wild-type activity in terms of both binding and activity was obtained when the two chimeras were co-expressed and so somehow something resembling the pure muscarinic or pure adrenergic structures, shown in Figure 8b, must have been generated. Elsewhere we have proposed that this occurred through domain swapping, as shown in Figure 8c (Gkoutos et al. 1999; Gouldson et al. 1997a, 1997b, 1998; Gouldson and Reynolds 1997; Kamiya and Reynolds 1999). This mechanism would suggest that helices 5 and 6 form the dimerization interface and this is certainly consistent

with Hebert's studies on inhibition by the helix 6 peptide (Hebert et al. 1996).

Other evidence that helices 5 and 6 are involved in the dimerization interface come from the observation that dimers of peptides from intracellular loop 3 of the α-adrenergic receptor are more potent activators of the G protein than the monomer (Gouldson and Reynolds 1997; Wade et al. 1994) (see below). Similarly, the observation that antibodies against adenosine receptors could not detect intracellular loop 3 in the dimer may arise from steric effects since the two copies of intracellular loop 3 are close together in the 5,6-dimer (Ciruela et al. 1995).

MOLECULAR DYNAMICS SIMULATIONS RELEVANT TO DOMAIN SWAPPING

The mechanism proposed in Figure 8c implies that if domain swapping can occur in Maggio and coworkers' functional rescue experiments, it could also occur during the normal activation process and, indeed, may be the normal activation process. It follows therefore that the 5,6-domain swapped dimer may be the active (high-affinity, R*) form of the receptor that interacts with the G protein. Consequently, we have investigated the energetics of this domain swapping process by carrying out molecular dynamics simulations (Grant and Richards 1995; Karplus and Petsko 1990) using the AMBER force field (Weiner et al. 1984, 1986) and software (Singh et al. 1988) on a variety of dimers and comparing their energetics in the presence and absence of ligand.

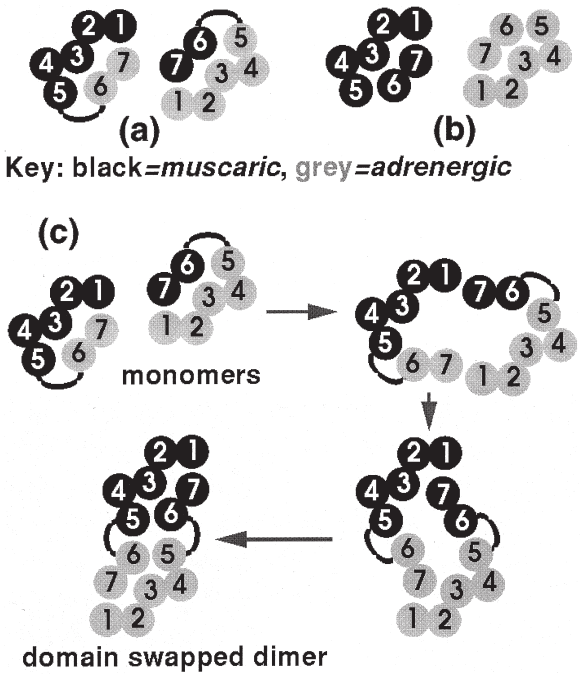

Figure 8. Domain swapping in G protein-coupled receptors (GPCRs). **(a)** the chimeric α_2-adrenergic-muscarinic M3 receptors used in Maggio's (1993a) co-expression studies; **(b)** the wild-type like receptors, presumable generated in the co-expression studies; **(c)** the domain swapping process proposed by Gouldson et al. (1997a, 1997b, 1998) to explain the functional rescue in Maggio's co-expression studies.

The dimers considered were the 1,2-contact dimer (that may be observed in some of the cryoelectron microscopy studies), the 1,7-dimer (a proposed intermediate on the domain swapping pathway shown in Figure 8c), and the 5,6-dimer—these structures are all shown in Figure 9 along with the relative average energies deduced from the molecular dynamics simulations. (Because the simulations were carried out on the transmembrane region of the receptor in the absence of loops, the 5,6-domain swapped dimer is identical to the 5,6-contact dimer.) As Figure 9a shows, the 5,6-(domain swapped) dimer is a high-energy structure compared to the two other representative dimers. It will, therefore, be present only in a very low concentration, in proportion to the Boltzmann population. This is in agreement with the low residual activity of GPCRs in the absence of ligand. Figure 9b shows that the 5,6-dimer is also a high-energy structure in the presence of one propranolol molecule, an antagonist (or even an inverse agonist). However, in the presence of one molecule of agonist the energy of the 5,6-(domain swapped) dimer is a low energy structure that is comparable to that of the 1,2-dimer—see Figure 9c.

Examination of the structures at the end of the molecular dynamics simulations suggests that the structural changes in the dimer mirror those in the monomer. Thus, the role of the agonist may be to stabilise the 5,6-dimer through conformational changes in helices 5 and 6—the very helices that form the dimerization interface.

These results are in good qualitative agreement with the mechanism shown in Figure 8c. However, these simulations were carried out on receptor models in the absence of membrane, solvent and receptor loops and so it is not possible to bestow these results with too much quantitative significance. Consequently, we have examined GPCR sequences to provide additional complementary information. Thus, in the next two sections we will examine the occurrence of both correlated mutations and conserved in class residues.

CORRELATED MUTATION ANALYSIS

Correlated mutation analysis played a useful role in confirming the value of the molecular dynamics-based docking studies. Initially, correlated mutation analysis was greeted with much enthusiasm as it was hoped that correlated mutations would be in close proximity and so could be used to predict protein structure from sequence (or rather from a multiple sequence align-

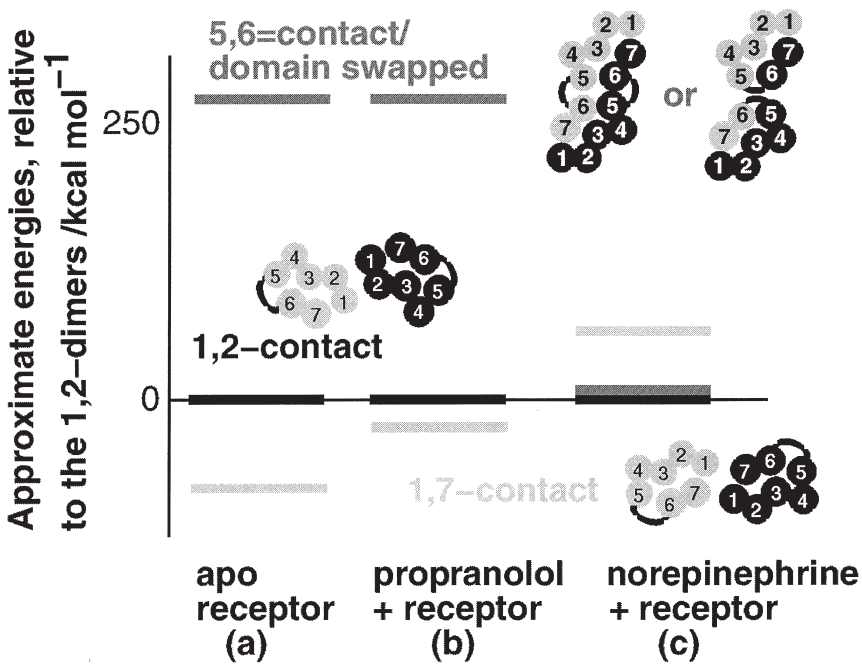

Figure 9. A schematic diagram to denote the average energies determined during the course of a molecular dynamics simulation on the 1,2-, 1,7-, and 5,6-dimers: **(a)** in the absence of ligand, **(b)** in the presence of antagonist (propranolol), and **(c)** in the presence of agonist (norepinephrine). The results are taken from Gouldson et al. (1998). (The energies in Figure 9a cannot be compared to those in 9b or 9c as different numbers of atoms are involved.)

ment). However, within the core of a protein there are many other ways, besides a compensating mutation, that a protein can use to mitigate against a potentially damaging mutation (Gouldson et al. 1998).

A protein-protein interface, however, may be more sensitive than the protein core. Indeed, Pazos and coworkers have exploited the tendency of correlated mutations to accumulate at protein interfaces in a novel approach to protein-protein docking (Pazos et al. 1997); this tendency is measured using the Xd descriptor, which is illustrated and defined in Figures 10a and 10b. When correlated mutations accumulate at the interface Xd is positive, whereas when ordinary residues accumulate at the interface Xd is negative. For the adrenergic receptors, correlated mutation analysis on ~50 aligned sequences has shown that the correlated mutations do accumulate at the 5,6-interface and Xd is indeed positive (Gouldson et al. 1998).

The external correlated residues, plus the external conserved residues at this interface are shown in Figure 10c. Correlated mutations have also been observed in external lipid-facing regions of the neurokinin and the opioid receptors—and here the correlated mutations are not restricted to helices 5 and 6 (Gouldson et al. 1997b). The conventional wisdom that the external lipid-facing residues are not functional and can therefore assume any identity as long as they are hydrophobic is seriously brought into question by these results.

EVOLUTIONARY TRACE ANALYSIS

Evolutionary Trace Method

The evolutionary trace method (Lichtarge et al. 1996a, 1996b, 1997), an adaptation of an earlier method of Livingstone and Barton (1993), is another approach to determining functional sites for a protein, given its X-ray structure and a multiple sequence alignment. It bears some similarities to correlated mutation analysis as the evolutionary trace residues may also be correlated but has the advantage that conserved residues are also included in the analysis. The basic assumptions of the ET method are as follows:

- that within a multiple sequence alignment, the protein family retains its fold—this is based on the idea that proteins that have evolved from a common ancestor will show similar backbone structure (Chothia and Lesk 1986);
- that the location of the functional sites is conserved;
- that these sites have distinctly lower mutation rates—because of the evolutionary pressures of residues defining the functional sites (Zvelebil et al. 1987);
- that this lower mutation rate is punctuated by mutation events that cause divergence.

The practical steps involved in identifying the evolutionary trace residues from a dendritic tree derived from a multiple sequence alignment are illustrated in Figure 11. The conserved residues correspond to the unbranched part of the tree on the left-hand side of Figure 11a, e.g., the conserved aspartate on helix 3 of β_2-AR (Asp^{307}) and the conserved serines on helix 5 (e.g., Ser^{504}). The first branch in the tree corresponds to the split between the alpha and the beta adrenergic receptors.

Residues such as 610 and 719 (Figure 11b) are termed conserved-in-class as they are conserved within the alpha receptors (as Val and Phe respectively) and within the beta receptors (as Ile and Asn), respectively but differ between the alpha and beta receptors. The next

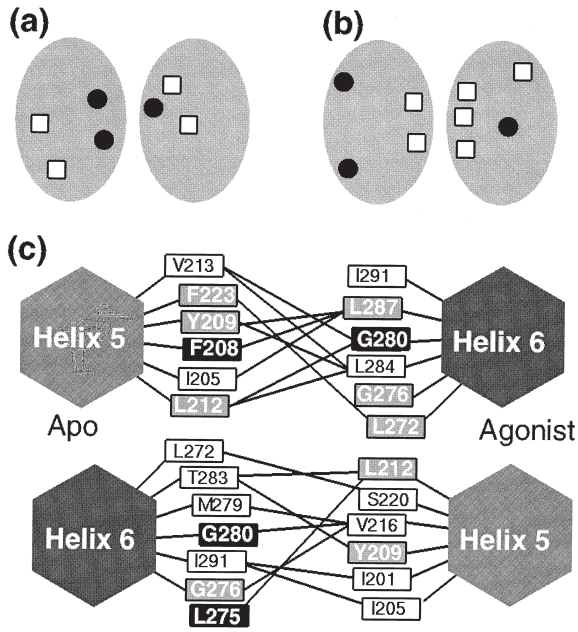

Figure 10. Correlated mutations at protein-protein interfaces. **(a)** and **(b)** illustrate the Xd descriptor (Pazos et al. 1997) which is positive if correlated residues (black circles, ●) preferentially accumulate at the interface. Xd is negative if ordinary residues (open squares, □) preferentially accumulate at the interface. **(c)** shows the accumulation of correlated mutations (white on gray rectangles) at the 5,6-dimer interface. The figure also shows the conserved residues (white on black rectangles) and ordinary residues (black on white rectangles). The residue-residue interactions observed using interactive molecular graphics are denoted by a black line; the structure was an average structure generated from a molecular dynamics simulation on a 5,6-dimer containing one agonist molecule. The Xd descriptor is positive for this system. The residue numbers correspond to those for the human β_2-adrenergic receptor.

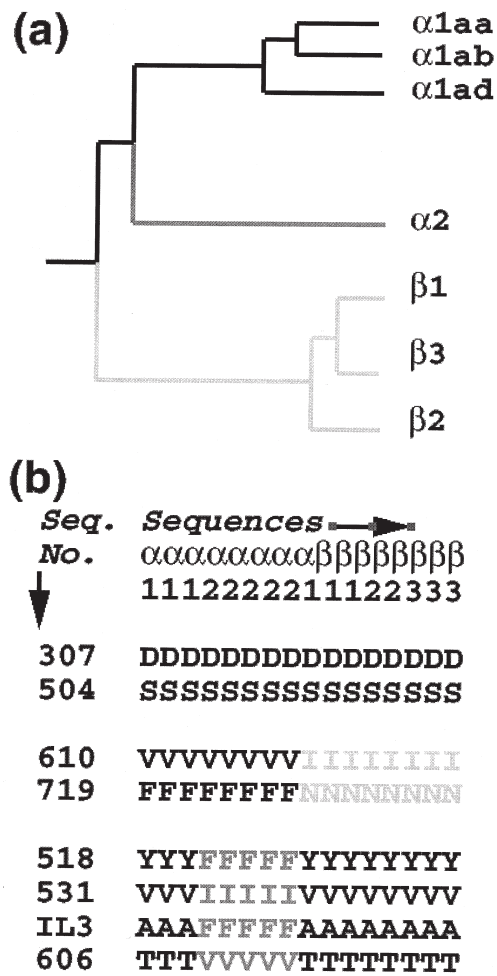

Figure 11. The evolutionary trace method. **(a)** (cut-down) schematic diagram of the dendritic tree for the adrenergic receptors; **(b)** sequence data at eight positions for 16 adrenergic receptor sequences (containing both α and β receptor types and 2–3 subtypes). Shading is used to illustrate the correspondence between the conserved-in class residues in **(b)** and the corresponding parts of the tree in **(a)**.

branch point corresponds to the split between the α_1 and α_2 receptors. Thus, 518 and 606 are also termed conserved-in-class as they are separately conserved within the α_1, α_2, and β receptors but are not necessary the same in any two classes.

The conserved residues and the conserved-in-class residues are plotted onto a space-filling model of the protein and in favourable cases they cluster around the functional site. At each step in the method, additional conserved in class residues are determined and plotted. At some point, addition of conserved-in-class residues from the more branched right hand side of the tree results in residues adding randomly rather than clustering around the functional site. In the earlier defining applications of this method (Lichtarge et al. 1996a, 1996b, 1997), this transition was determined by visual inspection, but we have used entropy and Monte Carlo envelope techniques (Upton and Fingleton 1985) to assist in this determination and the results will be reported elsewhere (Dean et al. 2000).

Here, we report a simpler statistical approach in which a cluster score was determined for the ET distribution and for 100 random distributions containing the same number of residues. The higher scores for the ET distribution, presented in Table 1, show that the distribution is clearly not random. There is certainly no shortage of sequence information on GPCRs for this analysis—there are for example over 300 sequences for the class A peptide GPCRs.

The External Functional Site on Helices 5 and 6

A number of interesting observations arise from the ET analysis on the various GPCR families. Firstly, a functional epitope is observed on helices 5 and 6 for each family and sub-family considered. Families considered to date include class A peptide, amine, opsin, nucle-

Table 1. A Comparison Between the Cluster Score for the ET Distribution and 100 Corresponding Random Distributions for Selected GPCR Families. A Score of +1 was Returned if a Residue had an ET Neighbour, Otherwise a Score of −1 was Returned. The Total Score was Determined by Summing Over All Residues—for Both The ET and The Random Distributions. The Number of Standard Distributions Between the Two Scores is also Reported. The Higher Scores for The ET Clusters Shows That The Clustering is Indeed Highly Significant. However, It Is More Appropriate To Evaluate The Level of Significance Using Monte Carlo Envelope Techniques.

Receptor Family	ET Cluster Score	Mean Cluster Score of 100 Random Distributions	No. of Standard Deviations Between Two Scores
Amines	70	52 ± 5	4
Nucleotide	20	9 ± 3	4
Olfactory	29	13 ± 3	6
Class A peptides	28	15 ± 4	4
Class B	37	21 ± 4	4

otide, olfactory, orphan, prostanoid, as well as class B and class C receptors (Dean et al. 2000). (The orphan receptors are not a well-defined sub family but rather a collection of poorly characterised receptors. Nevertheless, we have analysed them as if they were a well-defined family and they give similar results to the other GPCR families.) Representative results for the adrenergic receptor family are reported by (Gkoutos et al. 1999) for about 50 sequences. In the last 12 months, a further 10 sequences have become available and the evolutionary trace results are shown in Figure 12a. The agreement between these two sets of results (Gkoutos et al. 1999) and Figure 12a shows that they are not dependent on the precise identity of which adrenergic receptors have been sequenced to date. The evolutionary trace analysis does not yield exactly the same residues for each family, but the functional site is generally of the same size and in the same general region of the external face of helices 5 and 6. (In contrast, Figures 12b and 12d show that relatively few ET residues are observed on helices 1, 4, and 7.)

These results are in good agreement with the proposal that helices 5 and 6 form the dimerization interface, as discussed above—though the evolutionary trace analysis, as applied to the transmembrane regions of the receptor, cannot distinguish between contact and domain swapped dimers. Site-directed mutagenesis results on the external residues of helices 5 and 6 are not extensive but they do support a role for these residues in receptor activation (see Table 2).

The External Functional Site on Helices 2 and 3

In addition to the functional site on helices 5 and 6, Figure 12c shows that there is a similar site on helices 2 and 3. This result was somewhat surprising as the evidence for helices 2 and 3 participating in GPCR dimerization is not as strong as the evidence for helices 5 and 6.

With regard to domain swapping, extracellular loop 1 that connects helices 2 and 3 is generally much shorter than intracellular loop 3, which connects helices 5 and 6. However, in some receptors, such as the class B (secretin family) receptors, this loop may be relatively long (about 20–25 residues) and so domain swapping may be possible. Schöneberg and coworkers did co-express muscarinic receptor fragments split between helices 2

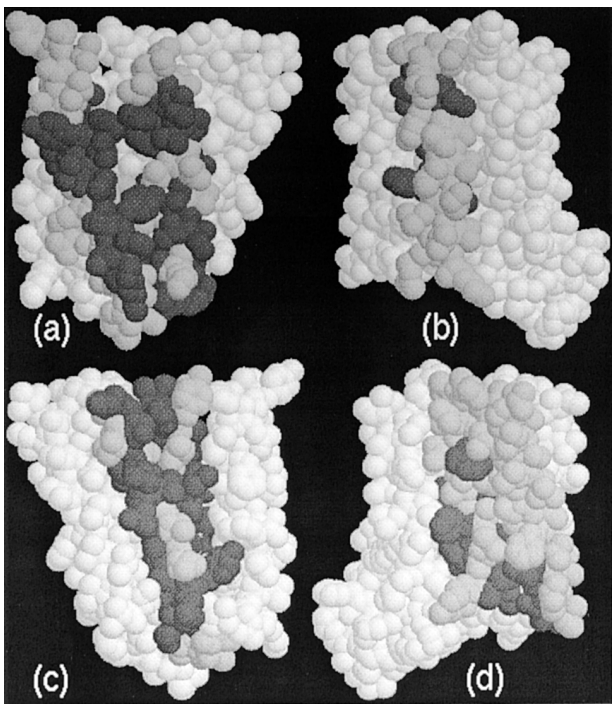

Figure 12. Evolutionary trace results for the transmembrane region of the adrenergic receptor family. **(a)** the functional site (dark grey) is plotted on helices 5 and 6 (light grey); **(b)** the ET residues (dark grey) on helix 4 (light grey) (these do not cluster to form a functional site); **(c)** the functional site (dark grey) is plotted on helices 2 and 3 (light grey); **(d)** the ET residues (dark grey) on helices 1 and 7 (light grey) (these do not cluster to form a functional site).

Table 2. Selected site-directed mutagenesis results on the external residues.

Mutation	Comments	Reference
Y205A(NK1) Y206A(NK2)	helix 5,—stops activity	(Huang et al., 1994b; Huang et al., 1995)
G276A G280A L284A	These mutations prevent Hebert's helix 6 peptide from inhibiting activation and dimerization	(Hebert et al., 1996)
W313A	helix 7, muscarinic, reduces maximum effect. This residue is borderline as regards being external—but it could affect formation of the 1,7-dimer as shown in figure 9	(Wess et al., 1993)
F222A	helix 5, δ-opioid, loss of activity	(Befort et al., 1996)
C69S	helix 2, muscarinic, high affinity binding abolished	(Savarese et al., 1992)
Q109H(NK1) H108Q(NK2)	helix 3, NK1—abolition of agonist (but not antagonist) binding	(Bhogal et al., 1994; Fong et al., 1992)
L116V(rat,NK1) V116L(human,NK2) M117L(NK2)	helix 3, NK1—antagonist binding increased or decreased	(Bhogal et al., 1994; Fong et al., 1992; Jensen et al., 1994; Sachais and Krause, 1994)
F112A	helix 3, NK2—agonist, antagonist binding increased	(Huang et al., 1995)
F115A	helix 3, rhodopsin, modest effects on signal transduction	(Nakayama and Khorana, 1991)
W126A,W126L, W126F	helix 3, rhodopsin, modest effects on signal transduction	(Nakayama and Khorana, 1991)

and 3 and they did not behave as autonomous folding units (Schöneberg et al. 1995). That is, the co-expressed fragments did not bind ligand or activate the G protein—unlike receptor fragments split between helices 5 and 6. However, extracellular loop 1 is particularly short in the muscarinic receptor and so these results may not be representative of those for receptors with longer loops. Thus, Schöneberg and coworkers' results do not rule out domain swapping in which extracellular loop 1 functions as the hinge loop.

Overall, the limited site-directed mutagenesis data does not support a functional role for the external residues on helices 2 and 3. However, there are reports of mutation effects that could be consistent with dimerization involving the external residues on helices 2 and 3 namely, affect on high affinity binding, abolition of agonist (but not antagonist) binding, increase in agonist binding and modest effects on signal transduction (see Table 2).

The functional site on the external face of helices 2 and 3, as identified by the evolutionary trace analysis, could be associated with functions other than homodimerization. It could be involved in GPCR heterodimerization (Jones et al. 1998; Jordan and Devi 1999; Kaupmann et al. 1998; White et al. 1998; Xie et al. 1999) or heterodimerization to proteins other than GPCRs such as receptor-activity-modifying proteins (RAMPs) (Fraser et al. 1999; McLatchie et al. 1998). It could even indicate sites for binding ion channels (Man et al. 1999) or other as yet unidentified proteins which need not be transmembrane. We note that yeast two hybrid studies using the $GABA_B$ C-terminal coiled-coil as bait detected many interacting partners (White et al. 1999), some of which (e.g., transcription factors) were totally unexpected.

Higher Order Structures

While there are no ready explanations for the origin of the second external functional site on helices 2 and 3, the presence of this second site could enable the GPCR to form higher order oligomers. Certainly, higher order oligomers have frequently been observed on gels (Zawarynski et al. 1998; Zeng and Wess 1999).

More relevant are the studies of Wreggett and Wells (1995) and Chidiac and coworkers (1997) who used mechanistic modelling to study co-operative binding of a series of antagonists to the muscarinic receptor. Wreggett and Chidiac's data was consistent with a receptor model containing four or more binding sites—since some receptors bind 2, 3, or 4 times more ligand than others. Given that a heptahelical receptor can only bind 1 ligand, the authors assumed that the data implied the presence of tetramers, or higher order oligomers. A tetramer formed by two 5–6 interactions and one 2–3 interaction is shown in Figure 13.

Similar observation of receptors binding variable amount of ligand have been observed by others. For example, Zawarynski and coworkers (1998) observed that the dopamine D2 receptor bound twice as much spiper-

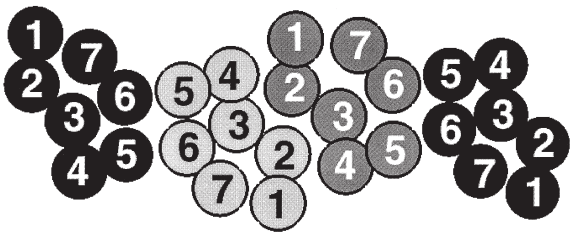

Figure 13. A Schematic diagram showing a GPCR tetramer formed by two 5,6-dimers interacting through the functional site on helices 2 and 3 (see Figure 12c). For reasons of clarity, each monomer is shown in a different shade.

one as benzamide. Wreggett and Wells (1995), Chidiac et al. (1997), and also Zawarynski et al. (1998) used antagonists in their studies and so they are observing higher order structures in the presence of antagonist. These observations appear to be in conflict with the idea that a significant proportion of dimers is formed only in the presence of agonist (see for example Hebert et al. 1996; Rocheville et al. 2000).

These observations of cooperativity and multiple binding modes in the presence of antagonists could result from GPCRs forming inactive dimers (and higher order structures) as well as conventional dimers. Moreover, the idea that a higher proportion of dimers in the presence of agonist does not necessarily predict that binding studies would detect anything other than single occupancy of an agonist within a higher order structure. The reason is as follows: if one molecule of agonist causes an optimal conformational change in helices 5 and 6 of one monomer such that the dimer is favoured, then a second molecule of agonist binding to the other monomer would cause a similar conformational change in the second monomer that will inevitably move the structure away from the optimum; consequently, the dimer would less favourable. Such structural changes could explain the origin of bell-shaped dose response curves that may be observed for G protein-coupled receptors (Jarv 1994, 1995; Jarv et al. 1995; Oras et al. 1999) and that are indicative of dimerization in other systems (De Meyts et al. 1995). Indeed, studies on changes in the population of dimers and higher order structures with concentration of agonist must take into account bell-shaped dose-response curves to guard against misleading results arising from the tail end of the curve.

The $GABA_B$ Problem

The recent reports that in the $GABA_B$ receptors system, heterodimerization was an essential prerequisite for function (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998), rather than an optional extra, certainly gave the GPCR dimerization story a new focus. The associated yeast two hybrid studies showed that the putative coiled-coil domain in the C terminus was essential for dimerization (White et al. 1998). This appeared to be a novel mode of dimerization for GPCRs and one that appeared to be in conflict with the more traditional view presented in our models, supported by Hebert et al. (1996) and Ng's et al. (1996) work, that the dimerization interface was contained in the transmembrane regions.

The part of the C-terminus N-terminal to the common coiled-coil region in $GABA_BR1$ and $GABA_BR2$ is however quite long (~24 and ~34 residues in $GABA_BR1$ and $GABA_BR2$, respectively). Consequently, interactive molecular modelling has shown that a coiled-coil interaction can be formed concurrently with a 5,6-transmembrane dimerization interface, as shown schematically in Figure 14. This structure was built on a foundation formed by joining the coiled-coil interaction found in the GreA X-ray crystal structure (Darst et al. 1994; Stebbins et al. 1995) to the adrenergic receptor dimer (Gkoutos et al. 1999; Gouldson et al. 1998). The interactive molecular graphics manipulations were carried out using the WHATIF modelling software (Vriend 1990).

Evolutionary Trace Analysis of the G protein

Lichtarge and coworkers have performed an evolutionary trace analysis of about 100 G protein sequences (Lichtarge et al. 1996b) and plotted the results onto transducin (Lambright et al. 1996; Sondek et al. 1996), the G protein that couples to rhodopsin. This enabled them to identify a *monomer* binding site on the G protein. In the light of our recent knowledge of GPCR dimerization, we have also applied our in-house evolutionary trace method to the G protein sequences and have found a functional site roughly twice as large as the one originally reported. These results will be reported elsewhere (Dean et al. 2000).

Clearly, a footprint for a dimer embedded in the G protein sequences provides evidence of a role for GPCR dimers in activation. However, this does not necessarily mean that the stoichiometry of the active complex is a two receptors to one G protein (Hebert et al. 1996) as the G protein could also form oligomers (Chidiac and Wells 1992; Green et al. 1997).

DISCUSSION AND CONCLUDING REMARKS

There have been many studies on the agonist-induced activation of GPCRs and while there is considerable agreement in the location of the conformation changes in the monomer that accompany activation, there is no general agreement on a unified theory that can explain all aspects of GPCR activation. Such a theory would have to explain agonism, constitutive activation, inverse agonism, partial agonism, antagonism, bell-shaped dose response curves, and functional rescue.

Figure 14. A speculative illustration of the $GABA_B$ heterodimer interaction. This figure (top) shows that the proposed coiled-coil interaction between the C-termini (White et al. 1998) can occur concurrently with dimerization involving the transmembrane regions. The structure has not been refined through molecular mechanics minimisation or molecular dynamics simulations so as to illustrate the complete lack of strain in this system. (Consequently the model does not yet assume the more compact structure associated with real protein structures.) The G protein heterotrimer (transducin) is shown below the $GABA_B$ dimer.

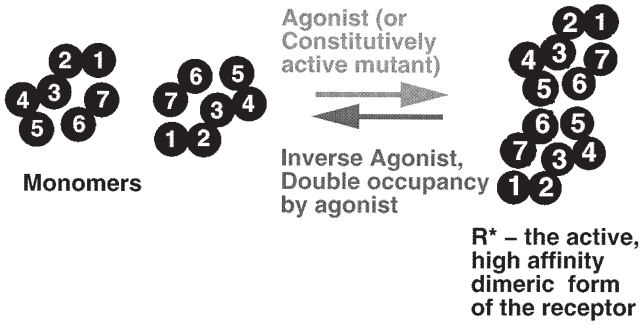

Figure 15. A proposed model of G protein-coupled receptor (GPCR) activation illustrating the conversion between monomers and active dimers, which may be promoted by agonists and constitutively active receptors and disfavoured by inverse agonists and double-occupancy of the dimer by agonist (which may be responsible for bell-shaped dose response curves). The dimer could be a contact dimer or a domain swapped dimer, but probably only domain swapped dimers could lead to functional rescue.

Dimerization may provide this unified theory and a possible scheme is shown in Figure 15. Implicit in this scheme is the idea that agonists promote dimerization and indeed parallel dose-response curves for signalling and ligand-induced dimerization been observed in some cases (Hebert et al. 1996; Rocheville et al. 2000). However, in other cases they have not been observed (Cvejic and Devi, 1997; George et al. 1998). One explanation for this disparity may be that the dimers are be pre-formed and merely rearrange in the presence of agonist.

The evidence for dimerization in GPCRs, indirect or otherwise, goes back many years (Avissar et al. 1983; Blum and Conn 1982; Conn et al. 1982a, 1982b; Hazum and Keinan 1985; Leiber et al. 1984), but despite the growing interest in GPCR dimerization over the last few years and despite parallel dose-response curves for signalling and dimerization, the definitive proof that homodimerization is required for activation has remained elusive.

Our theoretical studies into GPCR dimerization were initiated about six years ago as a result of visual inspection of the external transmembrane residues of a number of GPCR families using interactive molecular graphics, before we became aware of any literature on GPCR dimerization. We have since used three different theoretical approaches: molecular dynamics, correlated mutational analysis, and evolutionary trace analysis, and these concur in supporting the hypothesis that helices 5 and 6 are involved in the dimerization interface. The theoretical evidence that the 5,6-dimer is the *active* form of the receptor (R*) is less definitive as it is based only on the molecular dynamics simulations and the interpretation of experimental results obtained in other laboratories. Consequently, other roles for the external functional site on helices 5 and 6 must also be considered.

The functional rescue experiments on the co-expression of adrenergic-muscarinic chimeric (Maggio et al. 1993a) receptors provide the strongest experimental evidence for the involvement of helices 5 and 6 in the dimer interface and also for domain swapping (Figure 8), as suggested by Gouldson and coworkers (1998). Because the theoretical work was largely carried out on transmembrane receptor models in the absence of loops, it is impossible to discriminate between domain swapped and contact dimers from a theoretical basis. Currently, only domain swapped dimers can adequately explain the functional rescue experiments (Maggio et al. 1993b, 1996; Monnot et al. 1996).

Given the possibility of domain swapping in GPCR activation, it is appropriate to ask whether domain swapped dimers have any advantages over monomers or other dimers. Firstly, domain swapping is an evolutionary efficient method of forming dimers since the inter-domain interactions present in the monomer are re-used in the dimer interface. Secondly, domain swapping could help to minimize the effect of loss of function mutations—provided that two copies of gene expressed, that they are mutated in different domains, and most importantly, that the mutants do not adversely affect the free energy of folding. Thirdly, different dimers present the two copies of intracellular loop 3, which plays a key role in activation (Wess 1998), in different relative orientations, as shown in Figures 9. Possibly only one of these combinations may be correct for an active dimer—but see below.

Here, it is also appropriate to present the evidence against domain swapping. Hadac and coworkers (1999) used a photoaffinity analogue of CCK incorporating dual photolabile residues to probe the mechanism of the CCK8 receptor and found a covalent link between helices 1 and 7 of the *same* receptor, which would suggest that domain swapping is not happening. However, it is not clear whether this is a fair test, for several reasons. The photoaffinity probe was considerably less potent than CCK—it had a reported EC_{50} of 150 nM compared to 0.009 nM for the wild type CCK. The CCKA receptor has multiple affinity states (Huang et al. 1994a; Pandya et al. 1994; Talkad et al. 1994a, 1994b), only one of which may involve the dimer, and it is not clear which affinity state the photoaffinity probe detected. Moreover, CCKA(rat) has an unusually large charge of +9 on its intracellular loop 3 and this may permit activation by a receptor monomer (Dean et al. 2000)—the activation process is certainly believed to involve electrostatic interactions between the receptor and the G protein (Dean et al. 2000; Fanelli et al. 1999).

Schulz and coworkers (2000) have investigated the possibility of domain swapping in functional rescue by co-expressing full length V2 vasopressin receptors containing the R181C (helix 4) and the Y280C (helix 6) missense mutations. Functional rescue was not observed in this case and so this may indicate against domain swapping. However, vasopressin is X-linked and so two copies of the gene may not necessarily be available *in vivo.* However, Schulz and coworkers (2000) did obtain evidence for domain swapping in misfolded receptor fragments—though receptor fragments can never provide a full test of domain swapping as interactions present in the monomer cannot be fully re-used. In contrast to Schultz et al.'s studies, functional rescue was observed in the somatostatin receptor (Rocheville et al. 2000).

It is certainly interesting to consider why some receptor systems present evidence for domain swapping and others do not. Given the promiscuous nature of the GPCR-G protein association, it is possible that the GPCR can use a variety of modes, denoted as R* and R** (Schoneberg et al. 1999), to interact with the G protein. Alternative dimer structures, such as those presented in Figure 9, may contribute to this proposed diversity. It is equally possible, however, that the contact dimer and domain swapped dimer are actually equivalent as regards their ability to signal. While Figure 9 suggests that these dimers differ in the orientation of the two copies of intracellular loop 3, it should be noted that the N- and C-terminal portions of this loop contain helical extensions of the transmembrane helix.

The level of the helix extension varies according to the method, but is generally between 1.5 and 3 turns and is usually observed more strongly in the N-terminal region (Altenbach et al. 1996; Baldwin et al. 1997; Chopra et al. 2000; Gouldson et al. 1997a; Okamoto et al. 1991; Yeagle et al. 1997; Yeagle and Albert 1998). These regions, which are essentially perpendicular to the membrane in both dimers, are the most important part of intracellular loop three as regards signalling (Wess 1998). (Indeed, the activation of G proteins by amphiphilic cationic helices is well illustrated by the activation of G_o by mastoparan (Ross and Higashijima 1994) even if not by other such helices (Voss et al. 1993).) Thus, if the two dimers are structurally equivalent then the observed dimer, whether a contact dimer or a domain swapped dimer, will have the structure with the lowest relative free energy (or the one with the lowest barrier to formation).

In Maggio and coworkers' chimeras it is the domain swapped dimer that is likely to have the lowest free energy as it has the most native-like interactions but in dimers containing double missense mutants this may not necessarily be the case. Certainly, domain swapping equilibria may be significantly affected by even single mutations (Murray et al. 1995).

In addition to the 5,6-interface, the evolutionary trace analysis has identified another possible dimerization interface on helices 2 and 3. To date, we are unable to provide any theoretical evidence on the function of this second proposed external functional site, but a number of possibilities have been suggested and the theoretical results can be used to guide further experiments.

In summary, there is clearly much experimental evidence to show that GPCR dimerization occurs and that it is functionally significant. The precise structure and function of GPCR dimers, however, has not been determined. Here, it must be remembered that GPCRs undergo a range of interactions from their interaction with translocation machinery in the endoplasmic reticulum through their interactions with G proteins and kinases while on the cell surface to interactions with arrestin and other macromolecules involved in the internalisation process. Different oligomeric states may be in-

volved at different stages of this process. The theoretical studies presented here provide novel molecular level information that may be used to help investigate the role of GPCR oligomerization at each of these stages.

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