Dimerization of G-Protein-Coupled Receptors

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The evolutionary trace (ET) method, a data mining approach for determining significant levels of amino acid conservation, has been applied to over 700 aligned G-protein-coupled receptor (GPCR) sequences. The method predicted the occurrence of functionally important clusters of residues on the external faces of helices 5 and 6 for each family or subfamily of receptors; similar clusters were observed on helices 2 and 3. The probability that these clusters are not random was determined using Monte Carlo techniques. The cluster on helices 5 and 6 is consistent with both 5,6-contact and 5,6-domain swapped dimer formation; the possible equivalence of these two types of dimer is discussed because this relates to activation by homo- and heterodimers. The observation of a functionally important cluster of residues on helices 2 and 3 is novel, and some possible interpretations are given, including heterodimerization and oligomerization. The application of the evolutionary trace method to 113 aligned G-protein sequences resulted in the identification of two functional sites. One large, well-defined site is clearly identified with adenyl cyclase, β/γ and regulator of G-protein signaling (RGS) binding. The other G-protein functional site, which extends from the ras-like domain onto the helical domain, has the correct size and electrostatic properties for GPCR dimer binding. The implications of these results are discussed in terms of the conformational changes required in the G-protein for activation by a receptor dimer. Further, the implications of GPCR dimerization for medicinal chemistry are discussed in the context of these ET results.

Introduction

Evidence for G-protein-coupled receptor (GPCR) dimerization goes back many years,[1−9] but the recent reports of dimerization in the class A GPCRs (adenosine,[10] adrenergic,[11−14] angiotensin,[15] dopamine,[16,17] muscarinic,[18] opioid,[19] and vasopressin[11,20]), a class B GPCR,[21] and several class C GPCRs[22−28] indicate a renewed interest in the topic. It has been claimed that dimerization represents a revolutionary new concept in GPCR structure and function,[29] even though dimerization in other receptor systems is commonplace.[30] Moreover, because the human genome contains far fewer genes (∼30k−38k) than was expected a few years ago (∼50k−80k), it has been suggested that one of the ways in which the functional repertoire of proteins is controlled and enhanced is by protein−protein interactions rather than through additional genes.[31] GPCR dimerization and heterodimerization may offer a mechanism by which some of this control may be effected.

The position of GPCRs in cell signaling and trafficking ensures that they are attractive drug targets, and consequently, their dimerization may equally well present new opportunities in medicinal chemistry. The idea that dimerization depends on key sequence motifs is implicit in many of the published dimerization studies. Consequently, we have initiated a sequence-based study of the GPCR family to identify putative GPCR dimerization interfaces, and their associated G-proteins, to determine whether the corresponding interface on the G-protein is complementary to monomeric or dimeric receptor. Evidence that the G-protein binds dimeric receptors would make a significant contribution to the debate on whether dimerization is necessary for activation. We will also review the role of receptor domain swapping[13,14] in light of these and other results. The profound implications of these results for medicinal chemistry is further discussed, particularly in light of the recent studies on GPCR heterodimerization.

Two sequence-based studies suggest that our aim of identifying dimerization interfaces is achievable. First, Pazos observed that correlated mutations tend to concentrate at protein interfaces and used this observation to identify the correct docking orientation from several thousand incorrect orientations—for 21 protein heterodimers.[32] Second, the evolutionary trace (ET) method of Lichtarge et al., which is a development of an earlier

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method of Livingstone and Barton[33] and is essentially a data-mining approach to determining residues with significant levels of conservation, has been used to identify the SH2 and SH3 binding domains of tyrosine kinases.[34] For SH2 domains, the ET method had a specificity and sensitivity of about 90% and 70%, respectively (the figures for SH3 domains were lower). In our hands, the ET method has also identified the cytochrome *c* binding domain of cyctochrome *c* oxidase.[35] Pazos et al. used a minimum of 15 sequences, while Lichtarge et al. used about a hundred sequences, so the GPCRs are equally suitable for such studies because there are several hundred sequences for the amine and class A peptide families and at least 24 sequences for six other GPCR subfamilies; likewise, there are over a hundred sequences for the G-proteins.

The basic assumptions of the ET method are that within a multiple sequence alignment, the proteins share a common fold, that the location of the functional sites is conserved, that these sites have distinctly lower mutation rates, and that this lower mutation rate is punctuated by mutation events that cause divergence.[36,37] Here, we note that the GPCR superfamily members are characterized by a heptahelical fold and intracellular loops that are involved in G-protein coupling.[38−40] The superfamily is split into families and subfamilies according to function and specificity to the endogenous agonist.[41] Sequence similarity within these families is usually over the 35% threshold that allows automatic multiple sequence alignment to be used with confidence (at least for the transmembrane regions). Similar remarks also apply to the fold and sequence similarity of the G-proteins, and indeed, X-ray structures are available for both the receptor[42,43] and the G-protein.[44−46] Thus, both the GPCRs and the G-proteins are suitable for ET analysis. To date, there have been relatively few applications of the ET method.[47−51] However, the related correlated mutation analysis has been particularly successful for understanding GPCR structure and function.[52,53] Correlated mutations and ET residues cluster at the putative adrenergic dimerization interface.[14,54] Residues predicted by the ET method to be functional in regulator of G-protein signaling (RGS) proteins have been confirmed by mutagenesis,[48,49] and the ET results on the G-protein[55] are consistent with the N- and C-terminal regions, playing a major role in receptor coupling.[56,57] These studies lay the foundation for a more extensive ET analysis of the interfaces in GPCRs and G-proteins.

Results

The ET residues for the amine GPCR family, presented in Figure 1, show an ET functional site on helices 5 and 6 (Figure 1A), considerably less functionality on helices 1, 4 and 7, and a second ET functional site on helices 2 and 3 (Figure 1C). The same picture is generally reproduced across all of the GPCR families considered. Thus, Figure 2A−G shows the ET residues on transmembrane helices 5 and 6 of the peptide, opsin, prostenoid, olfactory, nucleotide, class B, and class C receptors, respectively, while Figure 2H−N similarly shows the corresponding ET residues on transmembrane helices 2 and 3. Tables 1−4 identify the positions of these external ET residues within transmembrane helices 5, 6, 2, and 3, respectively. From the table it is

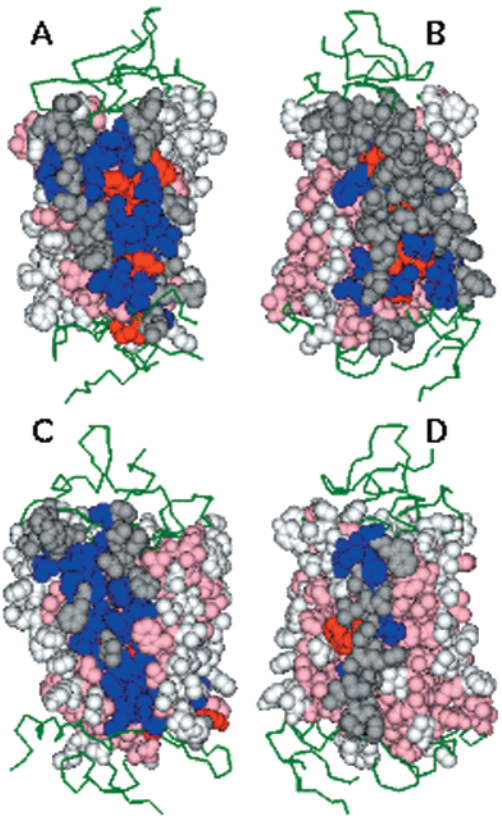


Figure 1. Four orthogonal views of the GPCR structure (PDB code 1F88) showing the ET results for the class A amine receptor subfamily plotted onto the transmembrane region. In (A), helices 5 and 6 are shown in gray; in (B), helix 4 is shown in gray, in (C), helices 2 and 3 are shown in gray, and in (D), helices 1 and 7 are shown in gray. The ET residues located on the gray helices are colored as follows: the conserved residues are shown in red, conserved-in-class residues are shown in blue. ET residues on the white helices are shown in pink.

clear that the ET method generally picks out about 50% of the external residues on TM3 and TM5 and 70% of the external residues on TM2 and TM6, albeit with variations between the different families. The highest proportion of ET residues is identified for the amine family, while the lowest proportion is identified for the peptide family. Generally, ET residues are identified at all external positions, though usually not in every family.

The probability that the ET distribution is not random, on the basis of the cluster score for neighbors defined by mutual contact to a water molecule, is given in Table 5. Generally, the percentage is high for each family, indicating well-clustered ET functional sites. The cluster scores were also determined by omitting helices 2 and 3 (to assess the quality of the functional site on helices 5 and 6) and by omitting helices 5 and 6 (to assess the quality of the functional site on helices 2 and 3). In most cases, but particularly in the amine and nucleotide families, the distribution remained highly nonrandom. However, the class B distribution became more random when helices 2 and 3 were omitted, while the olfactory and class C distributions became more random when helices 5 and 6 were omitted.

The ET distribution for the G-protein has already been published,[55] and the results relating to the receptor-binding site are reproduced in Figure 3a. We have

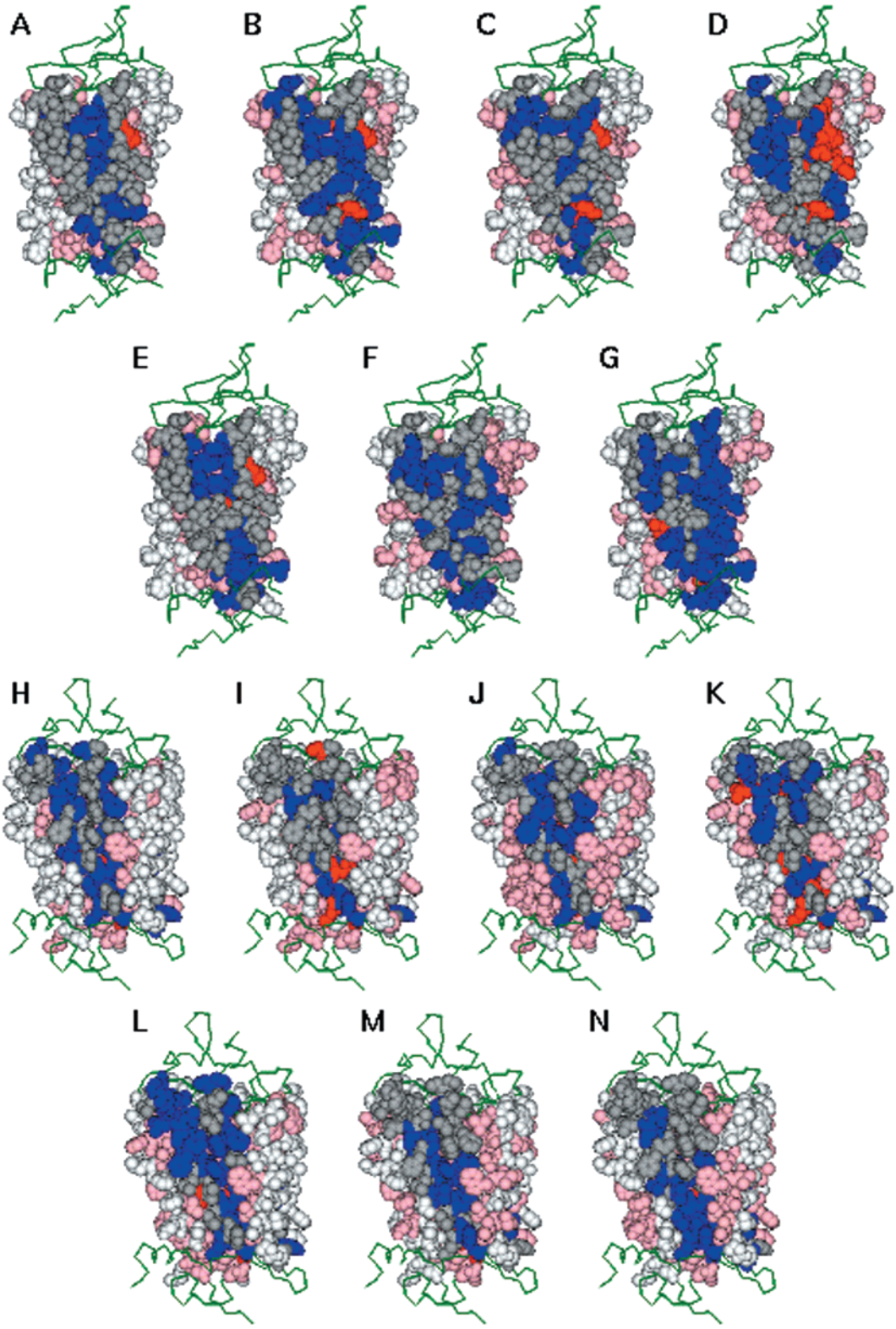


Figure 2. ET results for (A) peptide, (B) opsin, (C) prostanoid, (D) olfactory, (E) nucleotide, (F) class B, and (G) class C receptors. Helices 5 and 6 are shown in gray, but otherwise, the color scheme is as for Figure 1. ET results for peptide and class C receptors (H−N) are also colored as for Figure 1 except that helices 2 and 3 are shown in gray.

redetermined this distribution, and the results plotted onto the receptor binding and $G_{\beta\gamma}$ binding faces of transducin are shown in parts b and c of Figure 3, respectively. The electrostatic potential of the receptor binding face is shown in Figure 3d. Table 5 shows that the G-protein distribution is markedly nonrandom. The Monte Carlo envelopes for the G-protein shown in Figure 4 were generated with 49 lines, giving a 98% probability that this is not a random distribution. Similar data generated with 999 random lines (not shown) gave a 99.9% probability that the G-protein ET distribution is not random.

Table 1. Positions of ET Residues (Shown in Bold) on Transmembrane Helix 5, Conserved Residues (Shown in Bold Italics), and Non-ET Residues (Shown in Lower Case)[a]

No.	Opsin	Peptide	Amine	Olfactory	Nucleotide	Prostanoid	Class B	Class C
506	**E**	v	**P**	**E**	e	d	n	c
507	**S**	v	**A**	**L**	y	**A**	g	d
508	y	**Y**	**Y**	**L**	**M**	**A**	g	i
509	**V**	v	**T**	**I**	**V**	**F**	**L**	s
510	i	l	**I**	f	y	**A**	w	**D**
511	**Y**	l	**Y**	**I**	f	**L**	w	l
512	**M**	**L**	**S**	m	**N**	**L**	**I**	**S**
513	**F**	**F**	**S**	**G**	**F**	f	**I**	**L**
514	i	i	**I**	**S**	f	**A**	q	I
515	f	l	**G**	**L**	v	f	**G**	c
516	**H**	**G**	**S**	**I**	**W**	**L**	***P***	s
517	**F**	**F**	f	l	F	**G**	**I**	**L**
518	i	l	**Y**	**V**	**L**	**L**	**L**	g
519	i	l	**I**	**I**	i	l	**L**	y
520	***P***	**P**	***P***	**P**	***P***	**A**	**S**	s
521	l	**L**	**L**	**F**	l	**L**	i	g
522	i	l	l	**I**	l	**G**	**L**	l
523	v	i	**I**	l	l	a	**V**	***L***
524	**I**	**I**	**M**	**I**	**M**	**T**	***N***	m
525	f	a	l	l	l	**F**	**F**	v
526	**F**	v	**V**	**I**	g	**L**	**I**	**T**
527	**C**	**C**	**V**	**S**	i	**C**	**L**	**C**
528	***Y***	**Y**	***Y***	**Y**	**Y**	***N***	**F**	**T**
529	**G**	t	**W**	**V**	l	**L**	**I**	v
530	**Q**	**L**	**R**	**F**	l	l	**N**	**Y**
531	**L**	i	**I**	**I**	**I**	**T**	i	**A**
532	**V**	i	**Y**	**I**	**F**	**I**	i	**F**
533	c	r	r	s	r	l	r	k
534	a	t	a	s	a	a	i	t
535	v	l	**A**	i	l	l	l	r
536	k	k	r	l	r	l	v	g

[a] In each case, the residue shown is taken from the consensus sequence. The external residue positions are indicated by a gray background. The residue number given in the first column is the universal number taken from the GPCRDB.[59,60]

Discussion

General Comments. Elsewhere it has been shown that the ET method is able to predict protein−protein interaction sites. Thus, the observation that similar ET functional sites (Figures 1 and 2) are observed for all receptor families is significant because it indicates that each family has the potential to function in a similar way. These sites will be the fingerprints for interaction with complementary proteins in intramembrane macromolecular assemblies associated with the life cycle of the receptor and could indicate transmembrane dimerization (see above) or heterodimerization interfaces. (Heterodimerization has now been observed for receptor combinations involving adenosine,[68,69] angiotensin, bradykinin,[70] chemokine,[71] dopamine,[68,72] $GABA_B$,[26,28,73] metabotropic glutamate,[69] muscarinic,[74,75] opioid,[20] serotonin,[76] and somatostatin receptors.[72,77]) The ET functional sites could indicate regions involved in oligomerization prior to receptor uptake, regions for binding receptor-activity-modifying proteins (RAMPs)[78−81] ion channels[82] or other transmembrane proteins. Of all these possibilities, receptor dimerization is most consistent with the location of the ET sites; it has also received the most attention in the recent literature, and so our discussion will focus largely on this issue; other possibilities, however, are not ruled out by the data.

ET Results on the Receptor: The Interface on Helices 5 and 6. The results in Figures 1 and 2 suggest that there is an external functional site on helices 5 and 6. Elsewhere[14,83] we have discussed the evidence from the results of Maggio, Wade, Franco, Bouvier, and Huang in support of the 5−6 dimerization interface, a proposition consistent with the known role of helices 5 and 6 in activation (see, for example, ref 84). Here in

Table 2. Positions of ET Residues (Shown in Bold) on Transmembrane Helix 6, Conserved Residues (Shown in Bold Italics), and Non-ET Residues (Shown in Lower Case)[a]

No.	opsin	peptide	Amine	Olfactory	nucleotide	prostanoid	B	C
633		p	d	f	l	p	l	t
632	q	e	d	i	r	q	t	t
631	n	l	c	v	a	n	g	q
630	t	p	f	t	f	f	l	i
629	f	d	p	g	l	i	v	y
628	i	l	n	y	t	i	f	l
627	y	v	**V**	**F**	i	**K**	**E**	**K**
626	**F**	**D**	**L**	**L**	**C**	a	**D**	e
625	**A**	**L**	**N**	s	**N**	**M**	**P**	**A**
624	**V**	v	**T**	**V**	i	**R**	**V**	**S**
623	s	i	**I**	**V**	i	**V**	**F**	**Q**
622	**A**	**H**	**F**	**S**	**H**	**L**	**A**	a
621	***Y***	f	**F**	**L**	**L**	**L**	**F**	**T**
620	***P***	***P***	***P***	**H**	***P***	***P***	**V**	**G**
619	t	l	**L**	**S**	l	**S**	**V**	**F**
618	**W**	**W**	***W***	**A**	**W**	**W**	**Y**	**F**
617	**C**	**C**	**C**	**C**	c	***C***	**H**	**I**
616	i	l	l	**T**	l	v	**I**	**P**
615	**L**	f	i	**S**	a	**C**	**G**	**I**
614	**F**	**F**	***F***	**F**	***F***	**S**	**F**	**F**
613	a	v	**A**	a	l	**V**	**L**	**A**
612	i	l	**G**	**K**	f	c	**P**	**L**
611	**V**	**V**	**M**	**Y**	**L**	**M**	**I**	**W**
610	**M**	**V**	**I**	**K**	**V**	**I**	**L**	i
609	**V**	**V**	**I**	**G**	l	**G**	**V**	**I**
608	i	l	**G**	**E**	a	**A**	**L**	**C**
607	**V**	**V**	**L**	**V**	**L**	***L***	**T**	**T**
606	**M**	m	**T**	**S**	**S**	**Q**	**S**	**T**
605	**R**	r	**K**	**P**	**K**	**I**	k	**Y**
604	**T**	**V**	**T**	**I**	**A**	**V**	**A**	***M***
603	**V**	**A**	**A**	**K**	**A**	**M**	l	**T**
602	**E**	R	**K**	**L**	**K**	**E**	**R**	**F**
601	**K**	r	**K**	**I**	r	**V**	**R**	**G**
600	**E**	**K**	**E**	**S**	**E**	**E**	**Y**	**I**

[a] In each case, the residue shown is taken from the consensus sequence. The external residue positions are indicated by a gray background. The residue number given in the first column is the universal number taken from the GPCRDB.

Tables 1−4 we identify the key ET residues involved in these putative interfaces, and in Table 6 we present additional evidence from mutagenesis experiments that implicate many of these external residues in ligand binding, activation, correct folding, and disease.

The most frequently mutated residue is at position 517; this is an ET residue in all the families studied except the amine and olfactory families. The consensus sequence for the peptide family shows a phenylalanine at this position, and indeed, the majority of the residues mutated were phenylalanines or tyrosines. Mutations at this position show a variety of effects including the effect on agonist binding, antagonist binding, binding of agonist and antagonist, and effects on signal transduction. The most dramatic effect is the Y205A mutation in the neurokinin 1 receptor, which essentially abolishes both substance P binding and activation without effecting antagonist binding. Mutations at other positions also show effects on activation, but the causes may be different. Thus, the K602A and K605A mutations at the intracellular end of helix 6 may have a direct effect on G-protein coupling (though they could alternatively contribute under the positive inside rule) while the P620A mutations may effect the fold; this may be the cause of reduced cell-surface expression for a number of mutants. However, reduced cell-surface expression may also be linked to dimerization, as shown by the role of heterodimerization in the trafficking of both the

Table 3. Positions of ET Residues (Shown in Bold) on Transmembrane Helix 2, Conserved Residues (Shown in Bold Italics), and Non-ET Residues (Shown in Lower Case)[a]

Univ No	opsin	peptide	Amine	Olfactory	nucleotide	prostanoid	class B	class C
242		e	g	q	p	n	g	C
241	h	**G**	**M**	**S**	**G**	**S**	d	v
240	l	l	**L**	**L**	i	**A**	q	a
239	**S**	a	**E**	**F**	**S**	**Y**	s	t
238	t	**Y**	**Y**	**N**	**I**	**V**	g	d
237	**Y**	y	v	**A**	**Y**	**A**	e	p
236	m	a	**A**	**L**	**I**	**I**	**P**	k
235	**T**	**A**	**S**	**M**	**A**	**V**	**Y**	**A**
234	t	**W**	**F**	**K**	**L**	**V**	l	**I**
233	**T**	**F**	**P**	**P**	**P**	p	**V**	**M**
232	f	p	**M**	v	**P**	**S**	**A**	l
231	**G**	**L**	v	**T**	**L**	**T**	**D**	**F**
230	**G**	**T**	**L**	**V**	**L**	**L**	**K**	t
229	l	l	**L**	**S**	**L**	**L**	**I**	i
228	**V**	**A**	a	**S**	**G**	**T**	**F**	c
227	**M**	f	**V**	**F**	**V**	***G***	**V**	**Y**
226	**F**	l	**L**	**C**	**A**	**L**	**S**	c
225	**L**	**L**	**L**	**L**	i	**L**	i	**L**
224	**D**	**D**	***D***	**D**	***D***	***D***	**A**	f
223	**A**	**A**	**A**	**S**	**A**	**T**	**R**	**I**
222	v		**V**	**F**	l	**L**	**L**	**G**
221	**A**	l	**A**	**S**	***A***	**A**	**I**	a
220	**L**	**A**	**L**	**L**	***L***	***L***	**F**	***L***
219	**N**	n	**S**	**N**	**S**	**G**	**S**	**L**
218	**V**	**L**	**V**	**S**	v	**C**	**V**	**I**
217	**L**	**I**	**I**	**L**	**M**	**V**	**F**	**Y**
216	**I**	**F**	**F**	**F**	**F**	l	**L**	**S**
215	**Y**	i	y	**F**	**T**	**L**	**N**	**L**
214	**N**	**N**	n	**Y**	**F**	**L**	**M**	e
213	**L**	**T**	**T**	**M**	**T**	***F***	***H***	**R**
212	**P**	**V**	**V**	**P**	**A**	**S**	**I**	**G**

[a] In each case, the residue shown is taken from the consensus sequence. The external residue positions are indicated by a gray background. The residue number given in the first column is the universal number taken from the GPCRDB.

$GABA_B$ heterodimer and the RAMP−CRLR heterodimer.[78,85] Thus, the conformational properties conferred by the conserved prolines may play an important role in dimer formation. Mutation at position 619 causes *increased* rather than decreased activity. It is not clear whether this is related to its position immediately before the proline (the residue before a proline is known to affect the helix conformation[86]), to altered dimeric interactions, or to some other effect. For each of the mutations recorded here that affects activity, there are many others that have no observed effect. There are, though, many mutations that affect antagonist binding but not agonist binding and vice versa. There are also many mutations that affect binding of some ligands but not others, e.g., F196A and H260A in the MC1R receptor. The W276C mutation at position 511 in the endothelin B receptor has no effect on ligand binding, but it does result in decreased activation. These observations combine to ensure that the mutation record for the external residues is not complete unless binding and activation have been studied for a wide range of ligands. However, there is sufficient data in Table 6 to support the notion predicted by the ET method that these external residues are functionally important. It is clear too from the mutation record that there are functionally important residues that are not identified by the ET method; this is discussed in the section on the Monte Carlo results.

The ET results on helices 5 and 6 are in accord with two types of dimers: 5,6-contact dimers and 5,6-domain-swapped dimers. The basic domain-swapping mechanism to form a 5,6-domain-swapped dimer is shown in Figure 5A. A simple 5,6 contact dimer or heterodimer would use exactly the same 5−6 interface and would

Table 4. Positions of ET Residues (Shown in Bold) on Transmembrane Helix 3, Conserved Residues (Shown in Bold Italics), and Non-ET Residues (Shown in Lower Case)[a]

No.	opsin	peptide	Amine	Olfactory	nucleotide	prostanoid	B	Class B
311	**G**	**G**	**G**	**S**	**G**	s	n	d
312	**P**	n	**R**	y	**D**	**G**	t	t
313	t	a	v	**A**	h	r	v	a
314	**G**	l	L	g	g	**L**	**G**	v
315	***C***	**C**	***C***	***C***	***C***	c	**C**	c
316	n	**K**	**D**	l	**L**	t	**K**	s
317	l	l	i	t	f	**F**	**V**	l
318	**E**	v	w	**Q**	v	**F**	**A**	r
319	**G**	**S**	**L**	l	**A**	**G**	**V**	**R**
320	f	**F**	**A**	**Y**	***C***	**F**	**V**	l
321	f	l	**L**	f	**L**	t	f	f
322	**A**	**Q**	***D***	f	**F**	**M**	**F**	l
323	**T**	**Y**	**V**	**L**	**L**	**T**	**Q**	**G**
324	**L**	v	**L**	**L**	**V**	**F**	**Y**	**L**
325	**G**	**N**	**C**	**F**	**N**	**F**	**C**	g
326	g	**M**	**C**	g	**T**	**G**	**V**	f
327	**E**	**F**	**T**	d	**Q**	***L***	m	s
328	***V***	**A**	**A**	t	**S**	**S**	**T**	i
329	a	**S**	***S***	**E**	***S***	**P**	n	s
330	**L**	**I**	**I**	c	**I**	**L**	**Y**	**Y**
331	**W**	**F**	**L**	**F**	**L**	**L**	**F**	**S**
332	**S**	**L**	**N**	**L**	**F**	**L**	***W***	**A**
333	**L**	**L**	**L**	**L**	**L**	**G**	**L**	**L**
334	**V**	**T**	**C**	**A**	**L**	**S**	**L**	**L**
335	**V**	**A**	**A**	v	**A**	a	**V**	**T**
336	**L**	**I**	**I**	**M**	**I**	***M***	***E***	***K***
337	**A**	**S**	**S**	a	**A**	**A**	**G**	**T**
338	i	v	**L**	y	v	v	**L**	**N**
339	**E**	d	**D**	***D***	**D**	***E***	**Y**	r
340	***R***	***R***	***R***	**R**	***R***	**R**	***L***	l
341	**Y**	**Y**	**Y**	**Y**	**Y**	**C**	**H**	a
342	v	**L**	**W**	v	l	**L**	**T**	**R**
343	**V**	a	a	a	g	a	l	i

[a] In each case, the residue shown is taken from the consensus sequence. The external residue positions are indicated by a gray background. The residue number given in the first column is the universal number taken from the GPCRDB.

Table 5. Percentage Probability That the ET Distribution Is Not Random[a]

excluded	family	%	family	%	family	%
none	amines	99	olfactory	96	class B	97
	peptide	96	prostanoid	94	class C	99
	opsins	99	nucleotide	99	G-protein	99
TM2, TM3	amines	99	olfactory	97	class B	86
	peptide	99	prostanoid	94	class C	97
	opsins	98	nucleotide	97		
TM5, TM6	amines	99	olfactory	66	class B	98
	peptide	92	prostanoid	98	class C	82
	opsins	90	nucleotide	99		

[a] The percentage equates to the number of random cluster scores that are below the cluster score for the ET distribution.

differ only in the relative orientation of intracellular loop 3 (IL3), as shown in Figure 5B, because the domain swapping can in principle occur without any rearrangement of the A and B domains. Domain swapping was proposed by Gouldson et al.[13,14,83] as an explanation for the functional rescue observed on coexpression of Maggio's adrenergic−muscarinic chimeric receptors;[87,88] these experiments were originally performed to illustrate the ability of GPCR receptor fragments 1−5 and 6−7 to function as autonomous folding units. The ability of helices 1−5 and 6−7 to function as separate A and B domains has been shown for the rhodopsin,[89] adrenergic,[90] muscarinic,[91] vasopressin,[92] GNRH,[93] and the neurokinin receptors.[94] Such evidence for a dynamic structure reinforces the idea that these receptors could domain-swap. The possibility of domain swapping does not imply that domain swapping will necessarily occur in specific cases, so the relative merits of contact and domain-swapped dimers will be discussed further below.

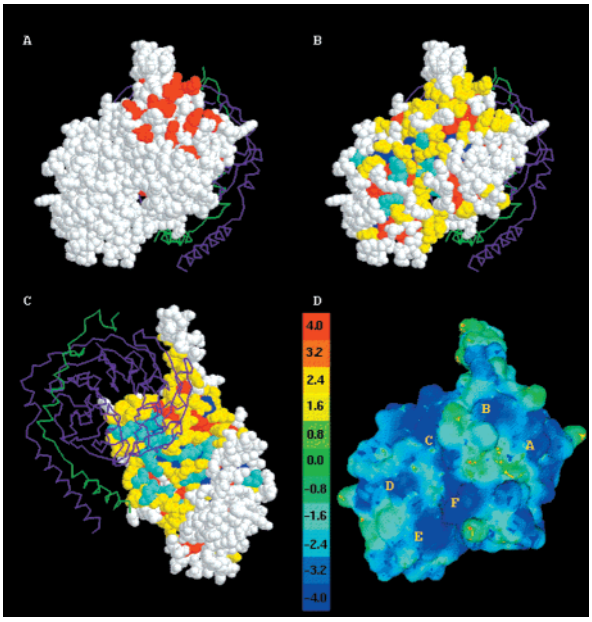


Figure 3. ET and related results for the G-protein: (A) the GPCR monomer binding site determined by Lichtarge et al., where ET residues are shown in red; (B) the GPCR binding site, from this study; (C) the $\beta\gamma$ binding face, where the $\beta\gamma$ subunit is also shown as a stick diagram. In (B) and (C) ET residues are colored as follows: external conserved and conserved-in-class residues are cyan and yellow, respectively; the corresponding formally internal but visible residues are blue and red, respectively. (D) Electrostatic potential (kcal mol^{-1}) is plotted onto the GPCR binding face. The more negative regions are labeled A−F.

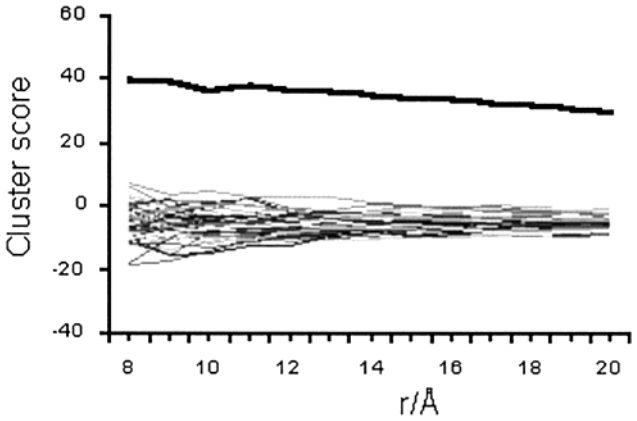


Figure 4. Monte Carlo envelopes showing the cluster score for the G-protein ET distribution (thick line) and the corresponding random distributions (thin lines) as a function of the distance cutoff used to defined the neighbors. The *x*-axis shows the cutoff radius used in the evaluation of the cluster score. The ET line is well above the 49 random lines, and so the probability that the distribution is not random is at least 98%.

These ET results suggest a unified mechanism for dimerization involving the transmembrane helices, despite the apparent alternatives (e.g., involving C-termini) reported in the literature. The report that $GABA_B$ receptors heterodimerize through an interaction involving their C-termini[73] is not in conflict with dimerization via transmembrane helices 5 and 6. Interactive molecular graphics has shown that a 5,6-dimer, as shown in Figure 5, can simultaneously allow the intracellular C-termini to interact via a coiled-coil interaction.[95] Indeed, it has also been shown that the $GABA_B$ heterodimer can form in the absence of the $GABA_BR1$ C-terminus.[96] However, the ease with which GPCR dimers can be identified may depend on additional stabilizing factors such as the coiled-coil interactions in the $GABA_B$ receptor[73] and the disulfide bridge interactions in the muscarinic[18] and the other class C receptors.[22,24] The involvement of Cys 140 and Cys 220[18] in stabilizing the muscarinic m3 dimer and multimer interfaces, as shown in Figure 1, is possible because they are located near the proposed functional interfaces on helices 2 and 3 (Cys 140) and helices 5 and 6 (Cys 220). However, the strongest stabilizing factor for a GPCR dimer could be its interaction with the G-protein. In addition, the implicit assumption in the ET method that the GPCRs share the same fold appears to be justified by the similarity in the results presented here for the various GPCR families and subfamilies (see also refs 61 and 97−99).

ET Results on the Receptor: The Interface on Helices 2 and 3. The functional site identified on helices 2 and 3, while not as well-defined as the site on helices 5 and 6, is novel in the sense that we are not aware of any experiments similar to Maggio's that clearly implicate the external face of helices 2 and 3 in activity. However, chimeric receptor studies have implicated the transmembrane region of helix 1, helix 2, or the intracelllular loop between them[80,100] as the site for interaction between RAMPs and the CLRC receptor. A role for helices 2 and 3 in dimerization has little support in the site-directed mutagenesis literature. Mutation of the external residues on helices 2 and 3 generally results in little or no effect on agonist binding, antagonist binding, or signal transduction. However, there are cases where a discernible effect is recorded when these external residues are mutated, including the abolition of high-affinity binding,[101] abolition of agonist (but not antagonist) binding,[102] increase in agonist binding,[103] and modest effects on signal transduction;[104] other effects are given in Table 6. Thus, a functionally important role for the external residues on helices 2 and 3 cannot be ruled out.

In the previous section, we considered 5,6-domain swapping as an alternative mechanism for 5,6-dimer formation, and so for consistency we also show a 2,3-domain-swapped dimer in Figure 5C. The work of Schönberg et al.[91] on the coexpression of muscarinic receptor fragments suggests that helices 1 and 2 (unlike helices 6 and 7) do not form an autonomous folding unit. This would tend to argue against domain swapping as a mechanism for 2,3-dimer formation but does not rule out contact dimers. Schönberg's muscarinic receptor contained a very short extracellular loop 1 (EC 1 joins helices 2 and 3), but there are several families of GPCRs where EC 1 is almost as long as IL 3, and so involvement of EC 1 in domain swapping cannot be ruled out. Since the hinge loop in a 2,3-dimer is extracellular, 2,3-domain-swapped and 2,3-contact dimers would interact with the G-protein in a very similar manner; the significance of this is discussed below.

The functional site on helices 2 and 3 could also be involved in heterodimerization to non-GPCR proteins such as RAMPs[78−81] and ion channels[82,105] as well as to other GPCRs. Indeed, yeast two-hybrid studies have shown that $GABA_B$ can interact with many macromolecules besides G-proteins.[25]

Table 6. Effects of Mutation on External Residues on Helices 2, 3, 5, and 6[a]

position	ref	mutation	receptor	effect
				agonist and antagonist binding
316	143, 144	**K102A**	AG2R	altered agonists and non-peptide antagonist binding
316	145	**K159A**	ET1R	altered agonists and non-peptide antagonist binding
517	146	**F226A**	P2Y1	agonist and antagonist binding
517	147	**F226A**	P2Y1	agonist and antagonist binding
517	148	**F222A**	δ-opioid	moderate effects on ligand binding
616	149	C283V	D1	2-fold reduction in agonist and antagonist binding
				antagonist binding
226	150	C80S	NK1	responsible for dramatic species selectivity seen with non-peptide antagonist WIN 51708
229	151	**F125S**	5HT2A	mesulgerine affinity decreased 10-fold
236	152	**M132L**	5HT2A	haloperidol affinity decreased 10-fold
316	153, 154	**D99N**	ACM1	decreased cooperative effects of gallamine binding twice!
511	155	T198A	angiotensin I	major role in antagonist binding
517	156	F198A	D2	no specific binding for [3H]N-0437; wild-type binding for other agonists and antaonists
517	157	**F227A**	CCKB	affect on antagonist binding
517	145	**F264L**	endothelin-A	increased binding of non-peptide antagonist bosentan
526	158	T198A	angiotensin II	affected antagonists but not peptide binding
608	159	**L390S**	muscarinic	affect on bindinng [^{3}H]NMS
623	160	V266I	NK-1	effect on affinity of antagonist CP-96345
624	161	**A313V**	A1AB	minor effects on antagonist binding
TM6	162	α1A → a1D	α1A-AR	majority of differences between α1A and α1D on TM6 are external. Could this be significant in subtype-specific ligand-binding properties?
TM6	84	X → Y	muscarinic	random mutagenesis study showing importance of various positions
				agonist binding
229	151	**F125L**	5HT2A	minor effects on binding of various ergots
240	163	H424A	FSHR	lower affininty for hormone binding, affinity for cAMP production increased
241	163	**T425A**	FSHR	increased binding, affinities for activation reduced
506	164	M200A	IL-8	significantly reduced IL-8 binding
510	165	H188A	PAF	impaired PAF binding, shift to low affinity state?
514	166	G261A	ETA	effect on binding of ET3
517	167	**F196A**	MC1R	affect on binding of selected agonists
517	103	**Y206A**	NK-2	reduced NKA binding
519	161	**V185A**	α1-AR	significant changes in agonist affinity
615	168	S268A	PGE2	loss of PGE2 binding
615	168	S268T	PGE2	altered agonist specificity of EP_1, EP_2, EP_3
619	156	L387A	D2	10-fold decrease in affinity for dopamine
619	166	F320A	ETA	effect on binding of ET3
624	167	H260A	MC1R	affect on binding of selected agonists
624	169	H260A	MC1R	binding affinity of α-MSH greatly reduced
626	170	**N283A**	NPY	small loss in affinity of NPY
				cell surface expression
321	15	S107A	AT1A	possible effects on cell-surface expression
619	171	**L286Y**	D1	reduced levels of expression
620	172	**P214A**	C5a	effect on binding and cell-surface expression
620	173	**P505A**	muscarinic	lower expression levels; binding, activation not affected
				activation
226	174	**F115A**	rhodopsin	altered activation kinetics
226	175	C454S	TSHR	slightly blunted functional activities, important for trafficing and expression
511	176	W276C	endothelin-B	reduced activity but no effect on cell-surface expression or ligand binding
515	175	C598S	TSHR	slightly blunted functional activities, important for trafficing and expression
517	177	Y205A	NK-1	no activation, reduced SP affinity, no effect on antagonist binding
517	178	**Y205A**	NK-1	binding of SP abolished, activation abolished
528	179	**Y216F**	NK-1	reduced activation of phospholipase C, loss of peptide discrimination capability, increased binding of some ligands,slightly reduced affinity for antagonists
602	180	**K362A**	muscarinic	did not stimulate cAMP production; lower levels of IP release, downregulated; impaired down regulation but normal internalization.
605	181	**K365A**	muscarinic	striking defect in coupling to PI hydrolysis
619	171	**L286A**	D1	enhanced basal and agonist induced activity, small binding affinity changes, shifts classical antagonists to partial agonists
620	174	**P267G**	rhodopsin	altered activation kinetics
620	174	**P267A/S/N**	rhodopsin	altered activation kinetics, reduced chromophore regeneration
627	174	**Y274F**	rhodopsin	altered activation kinetics
				miscellaneous
521	182	**C199S**	NK-1	site protected by agonist but not antagonist binding. Is dimerization an alternative explanation?
608	11	**G276A**	β2-AR	mutated TM6 peptide inhibits dimerization and activation
612	11	G280A	β2-AR	mutated TM6 peptide inhibits dimerization and activation
616	11	L284A	β2-AR	mutated TM6 peptide inhibits dimerization and activation
				mutations associated with disease
230	183	**G89D**	rhodopsin	retinitis pigmentosa: poor chromophore regeneration
510	184	**V194G**	D4	2 orders of magnitude less sensitive to dopamine, clozapine, and olanzapine; patient had low weight and no auxiliary or pubic hair; mutation is common in Afrocaribbean population.
612	185	F631L/C	TSH	somatic mutations associated with toxic thyroid nodules, possibly linked to constitutive activation
620	186	**P267L**	rhodopsin	retinitis pigmentosa: accumulates in ER rather than plasma membrane

[a] The ET residue positions are shown in bold. The receptors are referred to by name or mnemonic.

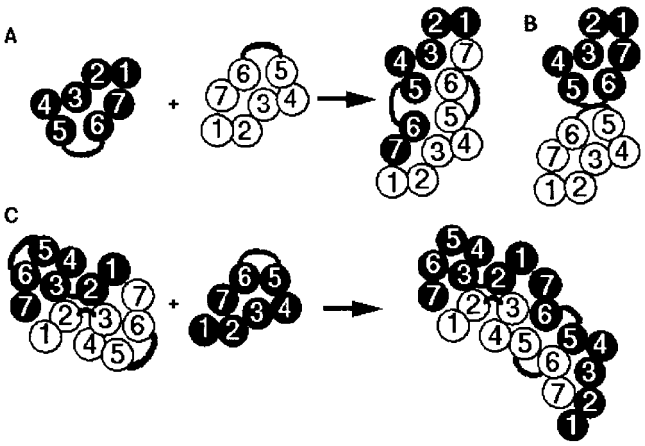
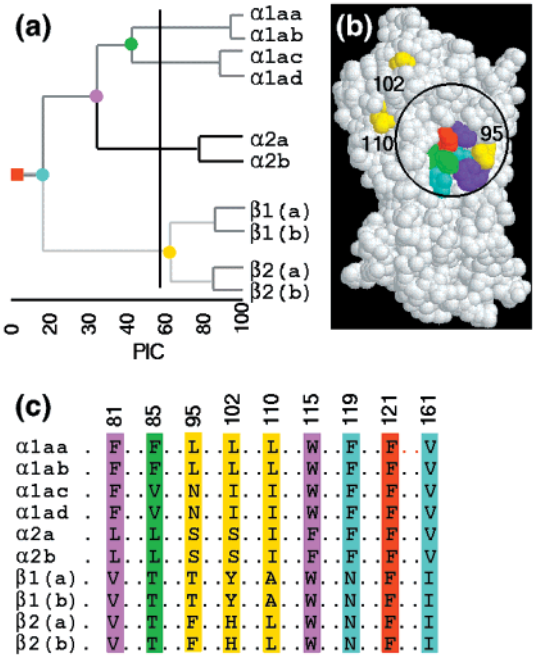


Figure 5. Possible oligomerization rearrangements in GPCRs: (A) domain swapping between helical domains 1−5 and 6,7; (B) a 5,6-contact dimer; (C) domain swapping involving a 2,3-domain-swapped dimer (left) and a monomer to form a domain-swapped trimer. A comparison of (A) and (B) shows that the domain-swapped dimer and the contact dimer helical arrangements differ only in the orientation of the interdomain loops. The two alternatives cannot be distinguished by the ET results.

Monte Carlo Results on the Significance of the ET Functional Sites. In general, Table 5 suggests that the ET distributions form distinct clusters because the cluster scores for the ET distributions are significantly above those of random distributions containing the same number of residues. However, some anomalous results emerge when either helices 5 and 6 or helices 2 and 3 are omitted.

For the class B receptors, when helices 2 and 3 are omitted from the calculations, the probability that the ET distribution is not random is only 86%. We are not aware of any well-documented reports of dimerization for this family, and so perhaps the tendency to dimerize is not so firmly coded in the sequences on helices 5 and 6 as it is for the other families. (An alternative explanation may be that class B receptors show *extensive* subtype-specific heterodimerization. A consequence of this would be that the residues controlling the subtype specific behavior would lie to the right of the ET cutoff (e.g., residues 95, 102, and 110; Chart 1a) and would therefore not be identified, though they would probably flank the core ET residues (see the discussion below on the peptide family). The class B site on helices 2 and 3 is significant however, and this may be correlated with the observation that RAMPs may bind near this region of the receptor to form a heterodimer that is involved in both trafficking to the cell surface and modifying the receptor activity.[78,85] Indeed, the firm heterodimeric association between this family and RAMPs may reduce the requirement for dimerization between two class B GPCRs. For the class C receptors, the ET patch on helices 5 and 6 is significant, but the ET patch on helices 2 and 3 is less significant (the probability that the distribution is not random is only 82%). One possible explanation consistent with this observation is that a functional site on helices 2 and 3 is not required for transport to the cell surface because other mechanisms (involving the N- or C-termini) are available. For the $GABA_B$ receptors it has been shown that the coiled-coil interaction between the C-termini is essential for normal trafficking to the cell surface; for the other class C receptors, disulfide bridges between the N-termini are important in dimerization and may also play a role in trafficking.

Chart 1. Illustration of the ET Method Using (a) a Dendogram Based on a Hypothetical Multiple Sequence Alignment, (b) a Space-Filling Structure Showing the ET Residues, and (c) Selected Residues from the Hypothetical Multiple Sequence Alignment



The peptide, opsin, and particularly the olfactory receptors also have ET patches on helices 2 and 3 that are less significant than those for other receptor families. Three observations apply to the peptide family and may be relevant to other families. First, external residues on helices 2 and 3 *do* figure in Table 6, indicating that these residues may be functionally important for the peptides. Second, the peptide receptors present the smallest number of ET residues even though their involvement in dimerization is clear. Third, for the somatostatin and opioid peptide receptors, subtype-specific heterodimerization has been observed for some subtype combinations but not others.[20,77] For these receptors, the ET residues are invariant between the subtypes that do not heterodimerize, and so the control of subtype-specific dimerization must reside with residues not detected by the ET method because the branch point in the dendogram lies to the right of the ET cutoff point (Chart 1a). For the detection of such residues, correlated mutation analysis may be more appropriate.[106,107] It follows, therefore, that if the ET method cannot detect all of the functionally important residues, then the scores in Table 5 may represent a lower limit.

Receptor Oligomerization. The general observation of two ET functional sites (helices 5 + 6 and helices 2 + 3, Figures 1 and 2) raises two possibilities: either the two sites have different roles (e.g., dimerization related to activation and dimerization related to trafficking) or they enable oligomerization. If the 2,3-dimer structure in Figure 5C (left) is possible, then contact dimer formation (or even domain swapping) is possible at two sites within the receptor, and the

possibility arises of forming trimers (Figure 5C) or even linear oligomers (not shown). There are now many observations of higher order oligomers (see, for example, refs 17 and 18), but the higher order structures receive little attention. Nevertheless, there are ligand-binding studies that show cooperativity and multiple binding effects that may be interpreted to infer dimerization, trimerization, tetramerization, and even higher order binding, even in the absence of G-protein.[108,109] Such higher order structures could not be formed without two functional sites, such as the ET patches shown in Figures 1 and 2. Our modeling studies do not suggest any reason for the higher order structures because the *dimer* contains the optimum-sized footprint for interacting with a single G-protein (see below), unless the G-protein also functions through oligomers.[110,111] Activation of GPCRs by antibodies (but not antibody fragments) and coupled antagonists (but not single antagonists) suggests there is no need to consider oligomers higher than the dimer in activation.[5,8,9] However, the alternative dimerization modes presented in our results do offer a possible mechanism for oligomer formation, and this may confer advantages in circumstances where receptor localization is important.

ET Results on the G-Protein. If the ET dimerization signals in the GPCRs are related to activation, then there should be a corresponding signal in the G-protein sequences. Prior to our study, Lichtarge et al. had already analyzed the receptor-binding region of the G-protein, as identified from GPCR peptide-mimetic binding,[44] and proposed a receptor-binding site[55,58] that is appropriate for binding a monomer (see Figure 3A). Here, we have reinvestigated this problem in light of recent work on GPCR dimerization, and our ET results are also presented in Figure 3. Comparison between parts A and B of Figure 3 shows that our ET functional site, and by implication the GPCR binding site, is approximately twice as big as was originally reported.[44,55,58] Before association of the whole of the Figure 3B site with GPCR dimer binding, it is important to assess whether the additional ET residues in Figure 3B are associated with binding sites for other proteins. Thus, Figure 3C shows that the other ET site on G_α binds the $G_{\beta\gamma}$ subunit. Significantly, X-ray crystallography shows that the $G_{\beta\gamma}$ ET site (Figure 3C) is also associated with adenyl cyclase and RGS binding.[45,46] This result significantly reduces the possibility that proteins other than GPCRs bind to the Figure 3B site.

The ET site in Figure 3B extends onto the helical domain of the G-protein; this is the region present in heterotrimeric G-proteins but absent in other G-proteins. The role of the helical domain has not been firmly established, but it is believed to modulate the activity of the ras-like domain.[112,113] Two pieces of evidence have been reported to suggest that the helical domain is *not* involved in G-protein coupling. First, no interaction was observed between the independent helical domain of transducin and rhodopsin.[112] Second, a chimeric G-protein containing largely the G_s sequence for the ras-like domain and the G_i sequence for the helical domain was coupled to the β-adrenergic receptor, which normally couples to G_s.[114] Recently however, an antibody to the *helical domain* of G_s has been shown to activate the G-protein in the same way as the receptor, providing novel experimental evidence for coupling between the helical domain and the receptor.[115,116] Moreover, some of the residues in the antibody binding site were identified in our ET analysis; these residues, along with other ET residues on the receptor-binding face of the G-protein and the experimental evidence implicating them in receptor binding, are given in Table 7.

Table 7. ET Residues on or near the Receptor-Binding Face of the G-Protein[a]

residue/ domain	region	residue/ domain	region	residue/ domain	region
N-term		**K313**[56]	α4-β6	***(I80)***[115]	αA
L15	α-Nt	Q314	α4-β6	***A83***[115]	αA
Q16	α-Nt	Y316	β6	***M84***[115]	αA
L19[57]	α-Nt	H318	β6	**L87**[115]	αA−αB
D22[57]	α-Nt	C321	β6	**R96**[115]	αB
A23[57]	α-Nt	**D324**[187]	α5	W127	αB
K25[57]	α-Nt	T325	α5	D129	αC−αD
D26[57]	α-Nt	N327	α5	G131	αD
A27[57]	α-Nt	**K329**[187]	α5	Q133	αD
T29	α-Nt	**D333**[56]	α5	R138	αD−αE
ras-like		**A334**[56]	α5	E141	αD−αE
I51	α1	**D337**[187]	α5	Y150	αE
I52	α1	**I340**[187]	α5	R157	αE
H53	α1	**K341**[187]	α5	Y163	αE−αF
S186	β2	**N343**[187]	α5	T166	αE−αF
F187	β2	helical		Q168	αF
L190	β3	G56	linker 1	L171	αF
K267	αG	S58	linker 1	R172	αF
D268	αG	***N72***[115]	αA		
I274	αG	***(S76)***[115]	αA		

[a] The residue numbers correspond to the positions in transducin, PDB code 1GOT. Residues in bold are implicated in receptor binding according to the associated reference number; several of these references identified a residue range rather than individual residues. Residues in bold italics are "below" the G-protein, and residues in parentheses are buried in transducin ($G_{\alpha t}$), but may be exposed in $G_{\alpha s}$ on which the experiments were performed.

There is, however, a steric problem in associating the whole of the Figure 3B site to receptor binding because the N-terminus is membrane-attached,[117−119] and it is impossible to satisfy this structural constraint and to keep the Figure 3B site facing the membrane. Analysis of the G-protein crystal structure containing RGS[45] suggests a resolution to this conflict because it shows a conformational change in the N-terminus (between Arg 28 and Val 30) that would enable the whole of the Figure 3B site to face the membrane while retaining the N-terminal membrane attachment.

The G-protein electrostatic potential is shown in Figure 3D. The spatial extent of the negative potential wells (sites A−F), which would be involved in the *long-range* attraction of the positively charged GPCR intracellular loops, is consistent with receptor dimer binding. The deepest negative wells are arranged near the central region; this is where the two copies of the functionally important IL3 would bind if the receptor were to bind as a 5,6-dimer (Figure 5). The positive electrostatic potential of the receptor intracellular loops may therefore contribute to G-protein activation by attracting the largely negative receptor binding face of the G-protein toward the receptor and the membrane. The concomitant conformational change in the N-terminus would also destabilize the interaction between G_α and $G_{\beta\gamma}$ (because $G_{\beta\gamma}$ is held between the N-terminal and the rest of G_α) by reducing the contact surface area and thus assisting the dissociation. In this model (Figure 6), receptor dimers, stabilized in the active

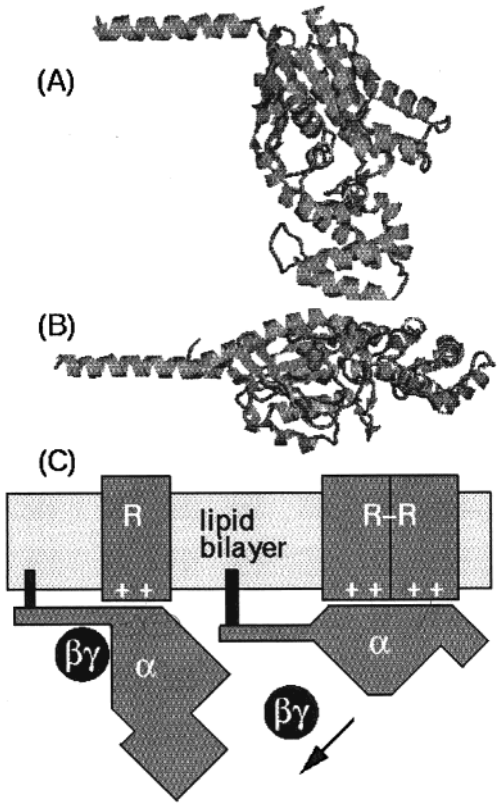


Figure 6. Mechanism of G-protein activation. (A) shows the conformation of the α-subunit of transducin in the heterotrimer structure,[44] while (B) shows the conformation in the complex with RGS.[45] (C), left, shows a schematic diagram of the G-protein in the same conformation as in (A) such that only half of the ET-derived receptor-binding site is near the membrane. (C), right, shows the schematic diagram of the G-protein in the same conformation as in (B) such that the whole of the ET-derived receptor-binding site is near the membrane.

conformation by ligand binding, would have a greater electrostatic attraction toward the G-protein through their greater net positive charge, but receptor monomers able to exert sufficient positive electrostatic potential could carry out a similar function. This model is highly speculative as regards the role of electrostatics in G-protein activation. However, with regard to the possibility of a receptor binding to the helical domain on the receptor binding face of the G-protein, it is firmly based on G-protein X-ray structural data[44] and receives additional support from the antibody binding data.[45]

Reports that $G_{\beta\gamma}$ also binds the receptor[120] are partially consistent with the ideas presented here because the membrane-facing surface of $G_{\beta\gamma}$ is "ET active" but its electrostatic potential is largely neutral (results not shown). The potential at the N-terminus of G_γ, however, is negative, and this may explain the binding of peptides derived from the receptor.[120] Taken together, the ET functional sites on G_α and G_β present an area that is more than large enough to bind a receptor dimer. The footprint of an inactive receptor monomer is about 40 Å, while the distance from the N-terminus of G_γ to the C-terminus of G_α is likewise about 40 Å. In addition, the distance from the C-terminus to the tip of the helical domain is about 75 Å. Moreover, the receptor undergoes considerable changes on activation, and so the footprint of the active form may be less than 40 Å.

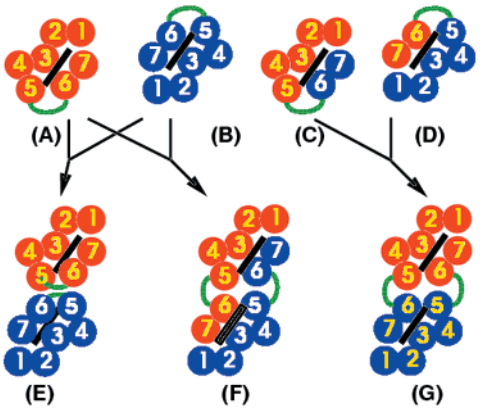
Nature of the GPCR−G-Protein Complex. Experimental evidence that dimerization is a necessary step in receptor activation is not entirely conclusive except for $GABA_B$,[28] though several studies present distinct functional changes that arise as a result of heterodimerization, including pharmacology,[20] internalization,[121] and G-protein coupling.[26,70,73] For example, parallel dose−response curves for signaling and ligand-induced dimerization have been observed in some cases[11,77] but not in others.[19,122] Taken in isolation, the receptor sequence data presented here does not permit a resolution of these issues except by directing further experiments. However, when the receptor and G-protein ET results are taken together, they provide new evidence that both the ras-like and the helical domain of the G-protein may interact with a receptor dimer and by implication that GPCR dimers are involved in G-protein activation. Further experiments will be required to determine whether the G-protein−GPCR dimer complex contains the stoichiometry necessary for activation under all conditions, and so discussion on whether the receptor forms a 5,6-contact or a 5,6-domain-swapped dimer is appropriate because each would present different regions to the G-protein, particularly in the case of a heterodimer, even though they are both consistent with the ET functional site on helices 5 and 6.

We are aware of two studies that present evidence against domain swapping as a general mechanism for receptor activation. A photoaffinity analogue of CCK incorporating two photolabile residues gave a covalent link between helices 1 and 7 of the *same* CCK receptor.[123] However, the CCKA receptor has multiple affinity states,[124−127] only one of which may involve a dimer, and it is not clear which affinity state the photoaffinity probe detected. Indeed, the photoaffinity probe had a reported EC_{50} value 4 orders of magnitude higher than that for wild-type CCK.

The most extensive investigation of domain swapping has been carried out by Schultz et al. who investigated whether functional rescue could occur when vasopressin receptors and receptor fragments containing missense mutations or truncations were coexpressed.[128] Functional rescue was not observed in all cases, and this may argue against domain swapping. However, Schulz did obtain evidence for domain swapping in selected cases, particularly when the extracellular disulfide bond was disrupted. (This is consistent with the observation that domain swapping in p13suc1, which is not a GPCR, involved a degree of unfolding.[129]) Even in cases where functional rescue was not observed, dimerization between the proteins was observed, and we may speculate that some combinations resulted in nonfunctional interactions; e.g., this could possibly occur between helices 2 and 3 and helices 5 and 6 mediated by the ET functional sites on TM2/3 and those on TM5/6. Functional rescue was also observed when two disfunctional somatostatin receptors were coexpressed.[77]

We propose that domain-swapped and contact dimers are essentially equivalent in their ability to signal, and this could underlie any failure to observe domain swapping. Experiment and sequence analysis have shown that both the N- and C-terminal portions of IL3 contain helical extensions of up to three turns beyond the lipid bilayer[13,98,130−134] and that these helical exten-

Chart 2. Formation of Contact Dimers and Domain-Swapped Heterodimers[a]



[a] The contact heterodimer, E, formed from A and B, contains native interactions with each transmembrane bundle. The domain-swapped heterodimer, F, may also be formed from A and B, but here both transmembrane bundles contain non-native interactions between helices from A and B. The domain-swapped dimer, G, formed between chimeric receptors, C and D, does, however, contain native-like interactions within each transmembrane bundle. In each helical bundle, a drug is shown interacting with helices 3, 5, 6, and 7.

sions are the most important part of the loop as regards signaling.[40] These extensions (but not the loop between them) are in equivalent positions in both the domain-swapped and contact dimers (see Figure 5A,B and Chart 2E,F). Thus, the observed functional dimer will have the structure with the lowest relative free energy (or the one with the lowest barrier to formation). This is discussed further in the next section.

Implications of GPCR Dimerization for Medicinal Chemistry. GPCR dimerization and heterodimerization may profoundly affect the way in which medicinal chemists approach ligand design. To begin with, the kinetics may be different for receptor dimers compared to the kinetics for monomers (the kinetics may be second-order in receptor "concentration", i.e., expression level, unless the dimers are preformed, as may be the case for the $GABA_B$[73] receptors). Second, it will be important to know whether the active form of the receptor is doubly occupied or singly occupied. (In the insulin receptor, double occupancy causes feedback inhibition of the receptor;[135] this kind of negative cooperativity could occur in GPCRs, and indeed, the occurrence of bell-shaped curves in many ligand−receptor activity measurements[136] is support for a dimer mechanism with feedback inhibition. On the other hand, significant positive cooperativity has been observed in the binding of MCP-1 and RANTES to the CCR2−CCR5 heterodimer.[71]) Other relevant issues are discussed below.

1. Design of Antagonists. GPCR dimerization may offer new opportunities for the design of antagonists because any ligand that inhibits dimer formation should act antagonistically. Indeed, this principle has been established using transmembrane peptides,[11,16] which are unlikely drug candidates. This approach to antagonist design is a challenge that the medicinal chemistry community is well equipped to confront and circumvent by designing the smallest and most "drug-like" compound that effectively mimics the peptide. Indeed, the ET sequence motifs in Figure 2A imply that for the peptide receptors it may only be necessary to mimic the top third of helix 6. Nevertheless, disrupting a protein−protein interface with a small organic ligand is likely to be thermodynamically challenging (for an example, see ref 137), and in the short term the design of traditional antagonists may be more fruitful.

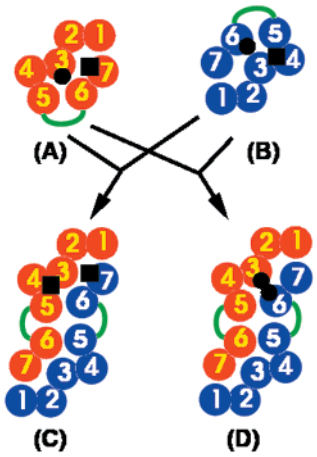
2. Heterodimerization and Drug Assays. The recent biochemical and pharmacological studies by Jordan and Devi on the opioid receptors have not only provided evidence for heterodimerization but also implied that the heterodimer exhibited novel pharmacology.[20] The implications of this for drug design can only become apparent when the full extent of the GPCR heterodimerization preferences are known. For example, we know that the dopamine D_2 and the somatostatin S_5 receptors are colocalized in neuronal subgroups and that they heterodimerize. The observation that dopamine ligands can affect somatostatin signaling pathways and vice versa implies that ligand binding and signaling assays should ideally involve not only the receptor of interest but also potential heterodimer partners.

3. Extent of Heterodimerization. The human genome probably contains about 600 GPCRs,[138] and although only a proportion of these may colocalize within a given cell, the potential number of partners may still be large. Our ET results should help in the design of experiments to verify and extend experimental studies on the extent of (hetero)dimerization. In somatostatin, heterodimerization occurs between S1 and S4 but not between S1 and S5. Since somatostatin is a member of the peptide family, the key ET sequence motifs on helices 5 and 6 are shown in Figure 2. The residues that enable subtype S1 to interact with S4 but not S5 are not shown in this figure because the ET residues identified are identical for S4 and S5; the same situation vis-à-vis heterodimerization applies to the opioid ET residues. In other cases of subtype-specific heterodimerization, the controlling residues may be identified by the ET analysis, but even if they are not, they are likely to flank the ET residues shown in Figure 2 (cf. residue 95 in Chart 1b). In either case, the ET residues could be used to guide molecular modeling and bioinformatics studies on the possibility of heterodimerization, which in turn may help in the design of more appropriate binary receptor assays.

4. Bivalent Compounds. There is a possibility of developing bivalent ligands for GPCRs; these could include linked heterodimeric compounds. These compounds would, in principle, offer a way to exploit GPCR dimerization for increasing affinity,[3,4] for synthesizing an agonist from an antagonist,[9] and for exploiting the novel pharmacology to arise from heterodimerization. Indeed, initial results on binary conjugates of adenosine A1 and A3 agonists have already been published.[139] Key design issues include the length and nature of the linker and its mode of attachment to the ligand. Even if bivalent compounds have molecular masses larger than those normally compatible with typical drug properties, they could still be used as tools to understand GPCR dimerization.[4,139]

5. Contact and Domain-Swapped Dimers and the Nature of the Heterodimer. This distinction between domain-swapped and contact dimers (compare

Chart 3. Functional Rescue through Domain Swapping[a]



[a] Receptors A and B, which are the same receptor but colored differently for clarity, each have 1 fatal mutation. For A this is on helices 3 or 7, and for B this is on helices 4 or 6. In C, the fatal mutations on helices 4 and 6 are swapped out to leave one intact seven-helical bundle. In D, the fatal mutations on helices 3 and 6 may be swapped out to leave one intact seven-helical bundle. However, this structure may not form if the new interactions involving the mutated residues are unfavorable.

A + B → E and C + D → G with A + B → F in Chart 2) becomes important when we consider some of the implications and opportunities for medicinal chemistry discussed above. For homodimerization where A and B in Chart 2 are identical, the preferred dimer, either E or F, will be the form most favored on thermodynamic or kinetic grounds. For receptors containing loss of function mutations, this is an important issue because domain swapping and the resultant functional rescue can only occur if mutations do not give rise to adverse interactions, as illustrated in Chart 3. Domain swapping may also require some degree of unfolding as proposed for the vasopressin receptor by Schultz[128] and shown for p13suc1.[129] The issue of adverse interactions is also important for heterodimerization because the domain-swapped heterodimer also appears to contain non-native interactions. As such, a key principle of domain swapping is violated, namely, that the interactions in the monomer should be reused in the dimer.[140−142] This violation makes structure F in Chart 2 unlikely for heterodimers. With this in mind, we should also note that Figures 1 and 2 are appropriate for considering the sequence motifs underlying heterodimer preferences in contact dimers (Chart 2E), since the results are plotted onto helices 5 and 6 refer to the same family. (For domain-swapped dimers, the heterodimer interface between receptors A and B, as shown in Chart 2F, essentially involves internal residues; the internal ET results are not shown, but they are given in Supporting Information and shown in ref 54 for the adrenergic receptors.)

6. Domain Swapping and Functional Rescue. Because domain swapping can result in functional rescue, it is a mechanism that may be exploited to treat certain genetic diseases. This may require some degree of unfolding, as shown by Schultz. She introduced mutations to prevent the formation of the extracellular disulfide bond and the resultant unfolding permitted domain swapping. Likewise, it may be possible to design compounds that upon binding give rise to sufficient unfolding to permit domain swapping and functional rescue.

7. Heterodimerization and Drug Design. A drug that interacts with a domain-swapped heterodimer, as shown in Chart 2F, would have to interact with helices from receptors A and B. It is a reasonable assumption that current drugs have been optimized to interact with seven helical bundles from a single receptor. The development of drugs for chimeric receptor bundles, as shown in Chart 2F, may offer new opportunities for trapping heterodimers. Indeed, Monnot's results[15] have been interpreted to imply that unusual domain-swapped forms can be trapped by favorable ligand binding.[14] Returning therefore to the dopamine−somatostatin heterodimerization, we note that heterodimerization raises the intriguing possibility that D_2 antagonists designed, for instance, to treat schizophrenia may be improved by including features in their design that would also encourage binding to the S_5 receptor. Chart 2F shows that there are at least two ways to do this, but there may be additional ways if the design also utilized the ET results. Although this may not actually enhance D_2 potency, it could be a valuable ally in encouraging specificity against other undesirable receptor subtypes.

8. GPCR−G-Protein Interface. Finally, compounds could be designed to target the G-protein−GPCR interface. Here, we have discussed the nature of the GPCR heterodimer, but exploitation of this particular drug design strategy would probably require further structural information. The ET results in Figure 4 could assist in designing experiments to determine this information.

Conclusions

In summary, we present here a study of the putative GPCR dimerization interfaces based on an enhanced ET method. We conclude that each family of GPCRs studied contains functional sites on helices 5 and 6 that are consistent with dimerization and that enable all the GPCRs studied to dimerize by a common mechanism. Although the results provide further evidence for helices 5 and 6 being involved in receptor dimerization, the data do not distinguish between a contact and a domain-swapped dimer. Indeed, contact and domain-swapped dimers may be equivalent in their ability to signal and their relative proportions (and hence the likelihood of observing functional rescue) are likely to depend on the their relative free energies. For this reason GPCR homodimers are more likely to be domain-swapped than GPCR heterodimers.

The putative receptor-binding site on the G-protein has also been identified, and this is consistent with receptor dimer binding in terms of both size and electrostatic properties. This ET site includes residues on both the helical and the ras-like domains; recent experimental evidence for the helical domain interacting with the receptor has been presented. The simultaneous interaction between the G-protein and both a GPCR dimer and the membrane would require a conformational change in the region where the α-helical N-terminus joins the main G_α ras-like domain. This conformational change would be similar to the one observed

in the G-protein−RGS X-ray crystal structure; it would weaken the interaction between G_α and $G_{\beta\gamma}$ and would thus help to initiate the release of $G_{\beta\gamma}$. Together, these results add to our understanding of GPCR dimers; they also provide important molecular information relevant to the GPCR dimer−G-protein interaction and hence the G-protein activation processes.

In addition to the functional site on helices 5 and 6, a novel receptor functional site was observed on helices 2 and 3. This site, along with the site on helices 5 and 6, could permit GPCRs to form higher order oligomers. It could also be involved in interactions with other proteins besides GPCRs.

Monte Carlo techniques were introduced for identifying the ET residues more rigorously, and indeed, it essentially confirmed that the ET residue distributions reported here were not random, though it did introduce some doubt as to whether class B receptors dimerize as readily as the other families. The probability that the G-protein ET site was not random was 99.9%. This Monte Carlo enhancement to the ET method may have applications in the determination of other medically important binding sites.

GPCR dimerization ard heterodimerization thus appear to be a property of the whole family rather than of selected receptors, and the many implications of this for medicinal chemistry have been discussed.

Methods

The basic approach for determining the ET residues follows that of Lichtarge et al.,[34,55,58] but we have additionally employed entropy and Monte Carlo techniques to determine when to terminate the ET analysis. The multiple sequence alignments for the GPCRs and the G-proteins were essentially taken from the GPCRDB, a GPCR sequence database.[59,60] Frimurer and Bywater[61] have presented a novel method for aligning the class A and class B receptors, and the same method was used here to align the class C receptors, including the $GABA_B$ sequences, to the class A receptors. The number of sequences used were 176 (amine), 240 (peptide), 112 (opsin), 32 (prostanoid), 39 (nucleotide), 31 (olfactory), 58 (class B), 24 (class C), and 113 (G-protein).

The procedure for identifying the conserved and conserved-in-class residues (referred to as ET residues) is illustrated in Chart 1. A UPGMA dendrogram (Chart 1a) was constructed from the neighbor module of phylip,[62] and the position of each node, along with its partition identity cutoff (PIC),[34] was determined. First, the conserved residues are identified (e.g., position 121 in Chart 1c). The tree is then analyzed on a node by node basis. After the first node (red), which has a PIC value of 20, the sequences are split into two classes, α and β, and so residue positions 119 and 161 are identified. Chart 1c shows that residue 119 is a conserved Phe within the upper α class of sequences and a conserved Asn within the lower β class of sequences. This procedure is repeated node by node. Thus, position 81 is the next to be identified. This is a conserved Phe in the α1 sequences, a conserved Leu in the α2 sequences, and a conserved Val in the β sequences. Residue position 115 is identified under the same criteria, while residue position 85 is identified as conserved in class at the next node (green). All of these residues cluster when plotted on a space-filling structure, as shown in Chart 1b. However, when the analysis proceeds by one more node, residues 95, 102, and 110 no longer add to the cluster (referred to below as an ET functional site) but rather appear to add randomly over the protein surface. Thus, a cutoff is defined between the green and yellow nodes, shown by the black vertical line in Chart 1a. In our full analysis, the results from several nodes were analyzed together, corresponding to increments of 10 PIC units; the cutoff was usually between PIC values of 70 and 80. In addition to the procedure shown in Chart 1, a small percentage error (∼3%) was permitted in determining the ET residues for some receptor families to ensure that the (almost) fully conserved DRY on helix 3 and NPXXY on helix 7 of the rhodopsin-like receptors were identified. Entropy, evaluated using eq 1, was used to eliminate random noise rather than ordered errors (such as ERY instead of DRY) at higher PICs; such ordered errors had lower entropy. (We also omitted high-entropy residues at high PIC values, as justified by the Monte Carlo method.)

$$S = \log\left(\frac{N!P!}{(P-V)!\prod_{i=1}^{V} M_i!}\right) \quad (1)$$

Here, N is the number of aligned sequences, P is the number of amino acid residue types (21 including gaps), V is the variability, i.e., the number of different amino acid types occurring at each position, and M_i is the occurrence of each amino acid type at that position. (In Chart 1c, N is 10, V is 3 for position 81, and M_i takes the values of 4 for F, 2 for L, and 4 for V at position 81). The logarithm was evaluated using the fuller form of Stirling's approximation.

In the original Lichtarge et al. articles,[34,55,58] the transition point between ordered clustering around the functional sites and random scattering over the surface was determined by visual inspection. Chart 1b shows the subjective nature of this step because residue 95 adds to the cluster, while residues 102 and 110 appear to add randomly. To minimize this problem, the probability that the final ET residue distribution was not random was determined using two Monte Carlo envelope-based techniques.[63] In the first technique, ET neighbors, lying within a defined radius of the α-carbon atom of a given ET residue, scored +1 while non-ET neighbors scored −1. (A 12 Å radius centered on the C_α of residue 121 is shown in Chart 1b. With a PIC cutoff of 50, the non-ET neighbors would include position 95 along with those that have not been colored.) In the second technique, external ET neighbors were identified by whether they could both contact (to within 2 Å) a water molecule (a sphere of radius 1.4 Å) rolled over the van der Waals surface of the receptor. In the first technique, the cluster score was calculated for the real ET distribution and 49 randomly generated distributions containing the same number of residues, and each score was plotted against the radius for a variety of radii ranging from 8 to 20 Å. The probability that the ET distribution was not random was determined by the position of the line joining the ET distribution relative to those joining the other distributions. Note that $^{49}/_{50} \equiv 98\%$, so if the line for the ET distribution lies above the 49 other lines, there is a 98% probability that the distribution is not random. If the ET line lies above 45 lines, then there is a 90% probability that the distribution is not random. In the second technique we simply determine 99 equivalent random distributions and note the number of random cluster scores above the score for the ET distribution. Both analyses were applied to the external residues only, whose identity was based on a relative exposed side chain surface area of 5% or more, based on an Ala-X-Ala tripeptide, as determined using the NACCESS program.[64]

In our analysis, the ET residues identified were plotted onto the (chimeric) transducin X-ray crystal structure[44] or onto the transmembrane region of the rhodopsin crystal structure.[42] The electrostatic potential was evaluated using the Poisson−Boltzmann method,[65] which includes solvent effects (by using a solute dielectric constant of 4 and a solvent dielectric constant of 78), and was displayed on the surface of transducin[44] using Gopenmol.[66] Formally, the electrostatic potential indicates the interaction energy with a unit positive charge, and so it gives a qualitative indication of where the intracellular loops of the GPCR are likely to bind because the positive inside rule[67] applies to the charges on these loops (though positive residues may reside on the intracellular loops for other reasons such as G-protein coupling).

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Supporting Information Available: A tabulation of the receptor and G-protein ET residues and multiple sequence alignments and a figure showing the cytochrome *c* binding site on cytochrome *c* oxidase, as determined using ET. This material is available free of charge via the Internet at http://pubs.acs.org.

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