

Oligomerization Domains of G Protein-Coupled Receptors

Insights Into the Structural Basis of GPCR Association

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1. INTRODUCTION

Many recent reviews have thoroughly described the ability of a wide range of G protein-coupled receptors (GPCRs) to exist and to potentially function as oligomers (1–6). This chapter summarizes the computational and experimental studies that have provided insight into the understanding of the structural basis of GPCR association. Particular emphasis is placed on the combined computational and experimental approach that we have recently developed to characterize the homodimerization interface of rhodopsin-like GPCRs.

Traditional models of GPCR activation have been based on the assumption that single agonists induce conformational changes in single receptors, which in turn stimulate heterotrimeric G proteins and produce signal amplification. However, recent reports on GPCR oligomerization have suggested that the ligands bind to and may activate an oligomeric complex (7–10), giving rise to various signaling events in the ensuing cascade. The requirement for GPCR dimerization in signaling has been demonstrated explicitly for the γ -aminobutyric acid (GABA)_{B1}–GABA_{B2} heterodimer. In this case, the subunit GABA_{B1} binds GABA but does not appear to be capable of G protein coupling, whereas the subunit GABA_{B2} cannot bind GABA but does appear to couple to G protein (8). Therefore, heterodimerization is a prereq-

uisite for receptor activation, which seems to occur through transactivation (8). It is also possible that for other GPCRs, the relevant unit defining their pharmacological characteristics may not be the monomer. Consequently, it is necessary to consider the structural details and modes of oligomerization to achieve an accurate understanding of receptor function.

Both computational and experimental efforts have been made to understand the basis of protein–protein interaction in GPCR oligomerization, but the specific molecular determinants required for receptor–receptor dimerization are still unknown. Additionally, there is still an issue regarding whether dimerization interfaces differ among highly related GPCRs. We have recently developed a combined experimental (11) and computational (12–15) approach to identify the molecular determinants responsible for GPCR oligomerization, with the goal of discovering mutations that disrupt the interface between protomers and, therefore, may interfere with receptor function. The computational approach produces putative three-dimensional (3D) models of oligomers based on the structural information contained in the crystal structure of rhodopsin (16) as well as on correlated mutation analysis (CMA) (12–15,17–21) that serves to significantly limit the number of different packing modes of the transmembrane (TM) bundles of GPCRs that must be considered in the modeling of oligomers. This approach has recently been applied to the three-cloned opioid receptor subtypes to identify their likely interfaces in both homo- (15) and heterodimers (13). Once the likely oligomerization interfaces were identified with the CMA-based approach, molecular modeling served in the construction of 3D models of GPCR dimers to maximize the number of interactions between the correlated residues that were predicted from CMA on the appropriate lipid-facing surface of the TMs in each protomer.

The computational procedure has recently been described for the opioid receptors (15). This chapter presents the results of CMA calculations performed on the other rhodopsin-like GPCR subtypes for which homodimerization has been experimentally demonstrated. To test these predictions, we have developed an experimental strategy that uses cysteine crosslinking to map the dimer interface of GPCRs. By applying this approach to the D2 dopamine receptor, we recently showed that TM4 forms part of a symmetric homodimer interface for this receptor (11). Interestingly, our CMA-based approach predicted TM4 as a likely dimerization interface for the D2 dopamine receptor. We propose to use the interdisciplinary approach described in this chapter as a tool to provide new insights into the understanding of the structural basis of GPCR oligomerization.

2. DOMAINS OF GPCR OLIGOMERIZATION INFERRED FROM EXPERIMENTS

To date, oligomerization of GPCRs has been suggested to be mediated by direct protein–protein interaction involving all the structural regions—extracellular, intracellular, and/or TM. A summary of the GPCR oligomerization domains that are experimentally suggested is provided later.

2.1. Extracellular Amino Terminus Domain

The involvement of the N-terminus in GPCR oligomerization has been clearly demonstrated for class C receptor subtypes. Direct evidence exists for the metabotropic glutamate receptor (mGluR)1 based on X-ray crystallographical data (22). Three different high-resolution crystal structures of the N-terminal ligand-binding region of this receptor, with and without the ligand, appeared as disulfide-linked homodimers. Several conformers of this amino terminus were identified by combining crystallographical data with modeling studies (22). Specifically, “active” and “resting” conformations resulted from interdomain movement and relocation of the dimer interface. Binding of glutamate to the extracellular ligand-binding domain of mGluR1 stabilized both the “active” dimer and a “closed” conformation of the protomer in dynamic equilibrium. The ligand-induced interdomain movements in the mGluR1 dimeric complex were suggested to produce an allosteric effect on the TM or intracellular regions of the receptor, leading to its activation.

A similar activation mechanism was recently proposed for the class C GABA_B receptor (23). Introduction of two cysteines, which were expected to stabilize the N-terminal ligand-binding domain of GABA_{B1} in a closed state by a disulfide bridge, locked the receptor into an almost fully active state.

Additional, although indirect, evidence for the participation of the N-terminal domain in GPCR oligomerization exists for other members of class C, as well as members of classes A, B, and D. Particularly, mutagenesis studies showed that cysteines within the N-terminal domain of the class C human extracellular calcium sensing receptor were critical for the formation of intermolecular disulfide bond(s) formed between receptor protomers (24,25).

The involvement of the amino terminus has also been implicated in the dimerization of the class A bradykinin 2 receptor (B₂R) (26). In the wild-type B₂R, the fraction of B₂R crosslinked in a dimeric or oligomeric form was increased by the binding of the agonist bradykinin, whereas this crosslinking was greatly reduced in a mutant that lacked the wild-type amino terminus and that started at amino acid 65, just before TM1 (26). Further-

more, the addition of a peptide corresponding to the amino terminus of the receptor reduced the amount of crosslinked B₂R dimers observed after bradykinin treatment, whereas peptides derived from the extracellular loops had no effect.

Western blot analysis of Ig-Hepta (a novel member of the GPCR superfamily defining a new subfamily of class B with a large extracellular amino terminus domain) indicated that this protein exists as a disulfide-linked dimer (27). The same analysis performed on the amino terminus domain of Ig-Hepta alone indicated that by itself, this receptor region lacks the ability to dimerize by forming disulfide bond(s). Nonetheless, experiments carried out on the mutant Ig-Hepta truncated after the first TM span indicated dimer formation, suggesting that the disulfide-linked dimer is formed through the cysteine residues in the extracellular domain, rather than in the seven-TM (7TM) helices. Assuming that the truncated Ig-Hepta mutants adopt a conformation similar to the extracellular domain of the wild-type receptor, these results suggest that although the covalent dimer is formed through intermolecular disulfide bond(s) in the amino terminus, TM1 is necessary for the molecular association of Ig-Hepta receptor.

Fluorescence resonance energy transfer (FRET) and endocytosis-based assays (which detect the ability of green fluorescent protein (GFP)-tagged endocytosis-defective receptors to interact with and be rescued by co-expressed untagged wild-type receptors) were used to analyze receptor deletion mutants of the class D yeast α -factor receptor sex pheromone exporter (STE)2 (28). These studies suggested that the α -factor receptor STE2 amino terminus, as well as TM1 and TM2, mediate receptor dimerization.

2.2. Intracellular C-Terminal Domain

Early indirect evidence suggested the involvement of the C-terminal region in the heterodimerization process of GABA_{B1}-GABA_{B2} (29,30) and in the homodimerization of δ -opioid receptor (31). In particular, yeast two-hybrid screening (29,30) showed that GABA_{B1} and GABA_{B2} subunits interact via a stretch of approx 30 amino acid residues within their intracellular C-terminal domains. Additionally, circular dichroism spectroscopy of polypeptide chain fragments containing the heterodimerization site of GABA_B receptor showed that these peptides preferentially form parallel coiled-coil heterodimers in a physiological buffer (32), suggesting that the functional GABA_B receptor is a heterodimer assembled by parallel coiled-coil α -helices contained in the intracellular C-terminal domain. Subsequent experiments with a series of GABA_{B1} receptor C-terminal truncation

mutants identified a sequence of four amino acids (RSRR) within the proposed coiled-coil interaction domain of the GABA_{B1} receptor that function as an endoplasmic reticulum (ER) retention signal in this receptor subunit (33). Therefore, the coiled-coil interaction with GABA_{B2} masks this ER retention signal and allows GABA_{B1} to come to the cell surface as a heterodimer with GABA_{B2}. Disruption of both the ER retention signal and the coiled-coil interaction domain allowed the GABA_{B1} receptor mutant to reach the cell surface, where it retained the ability to bind agonist but did not function. However, co-expression of this mutant with GABA_{B2} produced a heterodimer capable of inducing GABA-evoked G protein-coupled inwardly rectifying potassium (GIRK) current (33), demonstrating that regions (maybe TM helices) other than the intracellular C-terminal domain can mediate appropriate heterodimerization of GABA_B receptor subunits.

2.3. TM Domains

Dimer interfaces involving TM regions have been suggested for several GPCR subtypes, including rhodopsin receptors (34,35), β_2 -adrenergic receptors (ARs [36]), D1 (37) and D2 (5,11,38) dopamine receptors, C5a (39), α_{1b} -adrenoreceptor (40), and yeast α -factor receptors (41). As detailed below, the experimental approaches used atomic force microscopy and molecular modeling, synthetic peptides corresponding to various TMs, co-expression and FRET, and a strategy of disulfide-trapping of endogenous or substituted cysteine residues.

Specific oligomerization interfaces were suggested from molecular modeling based on the recently published atomic force microscopy analysis of rhodopsin. These pointed to the involvement of TM4 and TM5 in intradimeric contacts and to TM1 and TM2, as well as the cytoplasmic loop connecting helices TM5 and TM6, in the formation of dimeric rows (34,35). A 3D model of the rhodopsin homodimer was derived from these studies (Protein Data Bank identification code 1N3M [34,35]), offering predictions of specific interaction sites. Inhibition studies with a synthetic peptide corresponding to TM6 of the β_2 -AR suggested the involvement of TM6 in the dimerization of this family A GPCR (36). Specifically, a glycoporphin A-like dimerization motif (²⁷²LKTLGIIMGTFTL²⁸⁴) in TM6 of the β_2 -AR was hypothesized to play a role in the dimerization of this receptor. The use of synthetic peptides also identified TM6 and TM7 as possible dimerization interfaces of D2 dopamine receptors (38). In contrast, studies using the substituted cysteine accessibility method (42) and cysteine crosslinking experiments (11) suggested that TM4 forms part of a symmetric homodimer interface in the D2 dopamine receptor.

In contrast to the early findings for the β_2 -ARs (36) and D2 dopamine (38) receptors, a peptide based on the TM6 sequence of the D1 dopamine receptor did not affect the extent of dimerization (37), suggesting that the dimerization interfaces of closely related GPCRs could differ. Although the findings discussed earlier were shown to be specific for the sequences of these particular synthetic peptides, these data do not necessarily establish TM6 and/or TM7 as the dimer interface in β_2 -ARs or D2 dopamine receptors, because a specific peptide–receptor interaction at one site may modulate the ability of the receptor to form dimers at a different interface.

Specificity of oligomerization was also observed for GPCR heterodimerization (7,43,44), because some GPCRs were found to interact with one type of receptor but not another. For example, the μ -opioid receptor is known to heterodimerize with the δ -opioid receptor but not with the κ -opioid receptor. However, the κ -opioid receptor may form heterodimers with the δ -opioid receptor (43). Interestingly, the notion of selectivity in heterodimerization is also supported by computational analysis with the subtractive correlated mutation (SCM) method that we recently developed to identify likely heterodimerization interfaces among GPCR subtypes (13). Similarly to the opioid receptors, the somatostatin (SST)5 receptor has been reported to heterodimerize selectively, with SST1 but not with SST4 subtypes (7), whereas the chemokine receptor (CCR)2 has been demonstrated to associate with CCR5 but not with CXCR4 subtypes (44).

An interface involving TM1 has been proposed for C5a (39), α_{1b} -adrenoreceptor (40), and yeast α -factor (41) receptors. Thus, co-expression of the α_{1b} -adrenoreceptor with a fusion protein incorporating the N-terminal domain and TM1 of the α_{1b} -adrenoreceptor and $G_{11}\alpha$ was interpreted to indicate a role for TM1 in dimerization. Both TM1 and TM2 were suggested to form the interface of yeast α -factor receptor oligomers (28) based on the result of FRET experiments and assays showing that GFP-tagged endocytosis-defective receptors are recruited into the endocytic pathway through interaction with untagged wild-type receptors. Additionally, symmetric dimer interfaces involving TM1 and TM2 or TM4 of the C5 receptor were inferred based on disulfide trapping (39).

Taken together, the experimental results for various GPCR types suggest that TM1 and TM4 are the most likely interfaces for GPCR dimerization. Assuming a rhodopsin-like packing of the TM bundle for all GPCRs, TM1 and TM4 could not simultaneously participate in a single symmetric dimerization interface. Therefore, these results suggest either different dimerization interfaces for highly related GPCRs or the possible formation of higher order oligomers, as discussed below.

3. MODES OF INTERACTION BETWEEN TM REGIONS OF GPCR MONOMERS

Two modes of interaction between the TM helices of GPCRs have been proposed. First, “domain swapping” occurs when the TM bundles interpenetrate, and the interacting TMs from two different polypeptides appear as interlaced units (18,20,45,46). Second, “contact dimerization” occurs when each protomer TM bundle presents a separate binding site that is packed against that of another protomer through interactions at interfaces that would otherwise face the lipid environment. The domain-swapping mode of interaction was first suggested based on co-expression studies with mutant muscarinic and adrenergic receptors (47). Two chimeric constructs were used for this experimental study: α_{2C} -m3 and m3- α_{2C} , in which the TM6 and TM7 segments had been exchanged between the α_{2C} -adrenergic and the m3 muscarinic receptors. Although transfection with either of the two chimeric receptors alone did not result in detectable binding activity for muscarinic or adrenergic ligands, cotransfection with both α_{2C} -m3 and m3- α_{2C} restored binding of both ligands. This prompted the explanation that the TM1 to TM5 part of the receptor chimera formed a ligand-binding site using TM6 and TM7 from the second receptor chimera, thereby reconstituting a normal binding site comprised of TMs incorporated in two different polypeptide chains.

Although other examples of domain swapping have been proposed (19), more recent experimental results (28,34,35,48–51) have not supported this mode of interaction as a dominant form of GPCR dimerization but propose it only as a mechanism of functional rescue in heterologously expressed GPCRs. For example, nonfunctional point mutants and truncation mutants of D2 dopamine receptor were used to examine TM domain swapping in the oligomerization process of this receptor. Specifically, it was demonstrated that receptor function was antagonized when D2 dopamine mutant receptors that were incapable of ligand binding were expressed with the wild-type receptor (48), which is contrary to the expectation of reconstitution of intact binding pockets as a result of TM domain swapping. Additionally, no specific binding was detected upon co-expression of an Asp¹¹⁴Asn D2 dopamine receptor-defective mutant with a truncation mutant containing TM1 through TM5, suggesting that TM domains 1 through 5 do not participate in swapping with TM domains 6 and 7 in the D2 dopamine receptor. Similarly, nonfunctional constructs of the V2 vasopressin receptor with mutations in the N-terminal folding domain (TM1–TM5) could not be rescued by co-expressing a nonmutated N-terminal receptor fragment (51). Because all attempts to restore function of V2 vasopressin receptor mutants failed, but a

noncovalent interaction between monomers was detected by co-immunoprecipitation studies, oligomers of this receptor were also suggested to form by contact rather than by domain swapping. A recent study on the yeast α -factor receptor (28) also favored a dimer contact model over domain swapping: small fragments of this receptor (as simple as the N-terminal region plus TM1) could self-associate, unlike other receptor fragments lacking TM1. Intramolecular crosslinking between the TM1 and TM7 domains of m3 muscarinic acetylcholine receptor using an *in situ* disulfide crosslinking strategy did not produce dimers (49). In any domain-swapped dimer, regardless of the crossover point between the polypeptides, TM1 and TM7 (the first and last TM helices) must be from different protomers (unless multiple swaps are proposed). Therefore, crosslinking between TM1 and TM7 without the formation of a disulfide-bonded dimeric complex on nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting is strong evidence against domain swapping. Similarly, photo-affinity label experiments using a peptide agonist of the cholecystokinin receptor showed binding of this peptide to TM1 and TM7 of the same receptor (50), also arguing against the mechanism of domain swapping in the dimerization of this GPCR.

Finally, the recent atomic force microscopy map of rhodopsin (34,35), which shows receptor monomers organized into two-dimensional arrays of dimers, provides direct evidence for the formation of contact dimers, rather than domain-swapped dimers, in GPCRs.

4. AN INTERDISCIPLINARY APPROACH TO CHARACTERIZE THE OLIGOMERIZATION INTERFACE OF RHODOPSIN-LIKE GPCRS

Based on the preponderance of experimental data suggesting that the contact-dimer geometry is the most likely form of oligomerization among rhodopsin-like GPCRs, we recently developed a combined computational and experimental strategy to identify the molecular determinants for the oligomerization of rhodopsin-like GPCRs. The number of possibilities in which the 7TM regions of two GPCR monomers can be packed together is extremely large (at least $49 [=7 \times 7]$ for heterodimers and $28 [=7\{7 + 1\}/2]$ for homodimers). To reduce these possibilities, we recently developed two different computational approaches (6,13,15) based on a combination of CMA with the structural information of GPCR monomers derived from homology modeling, using the rhodopsin crystal structure (16) as a template. The computational method was used to identify likely interfaces of homodimerization (15) for all the rhodopsin-like GPCRs that are known to

dimerize to investigate whether oligomerization interfaces in different rhodopsin-like GPCRs are the same or different. The results from this study are reported below with a summary of the experimental method that we developed to test the computational predictions and characterize, in detail, the oligomerization interface(s) of GPCRs.

4.1. Dimerization Interfaces of Rhodopsin-Like GPCRs Predicted Computationally

Based on the principle that residues involved in a common function tend to mutate together, we recently searched for lipid-exposed correlated mutations within multiple sequence alignments of rhodopsin-like GPCRs for which homodimerization had been experimentally demonstrated. Specifically, calculations were performed for the following GPCRs: adenosine 1 receptors; angiotensin II type 1 receptors; α_{1b} -adrenoreceptors; β_1 -ARs; β_2 -ARs; B_2 Rs; cannabinoid 1 receptors; CCR2s, CCR5s, and CXCR4s; D1, D2, D3 dopamine receptors; H1, H2, and H4 histamine receptors; 5-HT_{1B} and 5-HT_{1D} serotonin receptors; leukotriene B₄-1 receptors; luteinizing hormone receptors; m2 and m3 muscarinic acetylcholine receptors; MT1 and MT2 melatonin receptors; neuropeptide Y type 1, type 2, and type 5 receptors; δ -, μ -, and κ -opioid receptors; SST2A, SST5, SST3, and SST1 receptors; thyrotropin-releasing hormone receptors; and V2 vasopressin receptors (3,40,52–55). Several receptors were excluded from analysis, including rhodopsin-like GPCRs with fewer than five full-length sequences from different species (CCR2, D3DR, H4R, L4R1, MT2, SST2A, SST5, SST3, and SST1) and GPCR subtypes for which the structural similarity with rhodopsin has been questioned (CB1, CCR5, and CXCR4) (56).

Rhodopsin-like GPCR sequences were retrieved from the GPCR database (57). Human receptor sequences were used as reference sequences for the multiple alignments of GPCRs, which were performed using the CLUSTALW program version 1.81 (58). TM regions were assigned based on the multiple sequence alignment of the entire rhodopsin family reported in the GPCR database (57). The 2.8 Å crystallographical structure of bovine rhodopsin (16) was used as a structural template in the definition of surface-exposed residues from 3D models (59,60) of the GPCR TM domains. Models of all receptors were constructed using the homology modeling approach implemented in the program MODELLER (61). Any residual steric repulsions between atoms of the side-chains in the resulting models were eliminated with mild energy minimization using version 27 of CHARMM (62). Specifically, 200 cycles of steepest descent followed by 200 cycles of conjugate gradient minimization were performed using a distance-dependent

dielectric constant of 4 ϵ and keeping all the backbone atoms restrained by harmonic potentials.

The sequence alignments and the 3D models were used in a computer program that identified lipid-exposed correlated mutations in GPCRs (6,15). This program builds on concepts embodied in general algorithms for the identification of correlated mutations (63,64). Lists of lipid-exposed correlated mutations for a given GPCR were extracted from the program outputs using the solvent accessibility values calculated from the atomic coordinates of each residue in the GPCR 3D models as criteria. Based on these values, pairs of correlated residues in which either one or both residues were inaccessible to lipid were eliminated from the lists. This pruning was performed to eliminate intramolecular contacts from the initial list of correlated mutations, which may have included predictions of both intra- and intermolecular contacts. Additional filtering criteria were applied to reduce the number of false-positives, although this procedure may produce some false-negatives by eliminating an actual interface from the resulting predictions. Therefore, the number of correlated pairs was first reduced to $L/2$, where L was the length of the GPCR sequence used as a reference in each multiple alignment. This filtering was performed because a list of $L/2$ was demonstrated to contain more correct predictions (64). Further eliminations from the $L/2$ list included any correlated pairs with a correlation index of 0.7 or less, which was done to reduce the number of false-positives. However, all correlated pairs with a correlation index equal to 1 were considered, even if the total exceeded the number of $L/2$ correlated pairs (*see above*). The residues that remained after filtering by these criteria were considered putative candidates for the interface of homodimerization of each GPCR under study. However, among these identified residues, an interface was considered to be predicted only if at least three were within seven residues from one another.

The constituent residues of the predicted structural neighborhood at dimerization interfaces (i. e., at least three lipid-exposed correlated mutations within seven residues from one another) of the rhodopsin-like GPCRs we studied are shown in Table 1. Notably, although calculations were performed for α_{1b} -adrenoreceptors, m2 muscarinic acetylcholine receptors, m3 muscarinic acetylcholine receptors, neuropeptide type 2 receptors, neuropeptide type 5 receptors, and κ -opioid receptors, no likely interface is reported because no residues satisfied the interface prediction criterion of at least three lipid-exposed correlated mutations close in sequence (within seven residues from one another). Because of the stringency of the criterion chosen to define dimerization interfaces and the filtering used to eliminate

most of the false-positives (*see* above), it is likely that some actual interfaces were not detected computationally.

Gouldson et al. (17,18,20) also carried out CMA calculations to identify likely dimerization interfaces of GPCRs. The main differences between those calculations and the protocol discussed earlier are: (a) the present method uses separate sequence alignments for each GPCR subtype, whereas Gouldson et al. did not separate subtypes and considered, for example, the entire family of biogenic amine receptors in a single sequence alignment and (b) additional criteria and filtering methods are incorporated into the present protocol to prune the original list of correlated mutations and to identify the dimerization interface neighborhood (e.g., the definition of likely dimerization interfaces based on the presence of at least three correlated mutations within seven residues from one another). Consequently, predictions from our CMA-based approach can focus on a small number of strongly predicted interfaces and involve only a few TMs, whereas the results from the larger alignments by Gouldson et al. (17,18,20) predict nearly every TM as a putative interface, most likely because they miss any putative subtype differences.

As demonstrated in Table 1, residues in TM1 and TM4 appear most often as putative interfaces among the studied GPCRs, making these TMs the most likely segments of rhodopsin-like GPCRs to be involved in oligomerization interfaces. This finding is intriguing, given the recent experimental data summarized in Section 1, which have suggested a role for these two segments in the dimerization/oligomerization of rhodopsin-like GPCRs, including rhodopsin receptors (34,35), D2 dopamine receptors (11), α 1b-adrenoreceptors (40), and C5a receptors (39). In particular, structural inferences from the recently published atomic force microscopy analysis of rhodopsin (34,35) have suggested that TM1 and TM4 form distinct symmetrical interfaces. The TM4 segment was specifically implicated in intradimeric contact between monomers, whereas TM1 was suggested to facilitate the formation of rhodopsin dimer rows. Despite the refinement and stringency of the CMA-based protocol described earlier, we believe that application of the CMA approach alone is not sufficient to exactly determine the entire correct interface for each GPCR, and additional computational efforts (such as the analysis of 3D models of GPCR dimers using the CMA predictions as a starting point) must be added for this purpose. Nevertheless, the agreement found thus far between CMA-based predictions with the stringent protocol and the experimental evidence regarding the likely interfaces (e.g., the preponderance of TM4 and TM1 in rhodopsin-like GPCRs, the subtype variability) underscores the usefulness and predictive

Table 1
Multi-Subtype Correlated Mutation Analysis of Rhodopsin-Like GPCRs

Res ^a	GPCR subtype																		
	AAIR	AGZR	BIAR	B2AR	BRE2	D1DR	D2DR	HIR	H2R	SH1B	SH1D	LHR	MT1	NY1R	OPRD	OPRM	TRHR	V2R	
1.30									A16										
1.31	F8								C17				L26						
1.32	Q9								K18				A27						
1.33									I19		I39								
1.34	A11																		
1.36																			
1.37											V43								
1.38	I15									V44			V33						
1.39									V24										
1.40	V17									S46									
1.41	L18								V27			L368							
1.44									L30										
1.45	V22								I31						V83				
1.47									V33				I42						
1.48	P25											I374	I43			F86			
1.51	V28											M375	L43			F89	I44		
1.52												T379							
1.55												F382	L50						
1.58	K35											L385				V96	M51		
1.59	V36																R52		
2.55														I91					
2.62														F98					
2.63					V83									V99					
2.64					A84														
2.66					I86														
2.67					A87														

Res ^a	GPCR subtype																	
	AA1R	AG2R	BIAR	B2AR	BRB2	D1DR	D2DR	HIR	H2R	SHIB	SHID	LHR	MTI	NY1R	OPRD	OPRM	TRHR	V2R
3.23										V120						II40		
3.24										V121						L141		
3.27										F124								
3.29																II46		
4.40		L143				K138			V133				K142		A163			A154
4.41		V144			A139													H155
4.43		K146			F141								L145					
4.44	V126							A146	II37						L167			
4.45																		
4.47													L149				II47	
4.48						V146	II58		L141	L172								V162
4.51	II33							F153				L492		V164	V174			A151
4.52	L134							L154						L165				
4.54										II78			L156	V167				
4.55	V137												A157	A168				
4.57																		
4.58												M499		L171			M158	
4.59	T141																L159	
4.60							L170											
4.61														F173				
4.62												V503	R164					
5.35		P192																
5.36			R222															
5.37			A223											D190				
5.40										II99				K193			L193	

Res ^a	GPCR subtype																	
	AA1R	AG2R	BIAR	B2AR	BRB2	D1DR	D2DR	HIR	H2R	SH1B	SH1D	LHR	MT1	NY1R	OPRD	OPRM	TRHR	V2R
5.41		T198	A227									I533		D194			M194	
5.43														Y196				
5.44		I201									C203		V193		L219			
5.45												V537					V198	
5.47														D200				
5.48													L197				V201	
5.49		F206									I208	I541	V198					
5.51														S204	I226			
5.52	L194								I197									
5.53														S206				
5.55	V197								I200				I202					
5.56	L198												I204					
5.58									Y203				F205		V231			
5.59	L201																	
6.30		N235																V266
6.31					R266													
6.32					R267													
6.34																		V270
6.35																		R271

Res ^a	GPCR subtype																		
	AA1R	AG2R	BIAR	B2AR	BRB2	D1DR	D2DR	H1R	H2R	5H1B	5H1D	LHR	MTI	NY1R	OPRD	OPRM	TRHR	V2R	
6.36		I241																	
6.39					V274								V242						
6.42		L247			L277														V278
6.45					I280														
6.46																			
6.49																			
6.53																			
6.55											S321								
6.57																			
6.58																			
6.59											L324								
6.60											P325								
7.33																			
7.34																			
7.36																			
7.37																			
7.41																			
7.40																			
7.41																			
7.44																			

^aResidue numbers refer to human receptor sequences. The generic numbering scheme for GPCR sequences (73) is also used to make possible comparisons among the different GPCRs.

power of the approach. Notably, the computational method is also capable of providing useful information about conserved interfaces in oligomeric assemblies larger than dimers.

4.2. Testing and Validation Using Cysteine Crosslinking Experiments

Numerous studies using crosslinking have demonstrated that GPCRs in the membrane are dimeric or oligomeric complexes (26,31,36,65–67). These studies used relatively long, lysine-reactive bifunctional crosslinking reagents, which made it impossible to infer the specific residues or regions that were crosslinked. To directly identify the dimer interface, we designed a strategy to use cysteine crosslinking (11) of our collection of D2 dopamine receptor-substituted-cysteine mutants (59,68). It was essential to develop a system that would allow a non-crosslinked receptor to run as a monomer on nonreducing SDS-PAGE. Our background construct FLAG-D2 dopamine receptor (11) ran almost exclusively as a heterogeneously glycosylated monomer of approx 65 kDa on nonreducing SDS-PAGE (Fig. 1A). Therefore, if this D2 dopamine receptor is oligomeric, the oligomer dissociates in SDS. Additionally, unlike some class C receptors, the D2 dopamine receptor is not an obligatory disulfide-linked dimer in the plasma membrane (69,70).

As a control before introducing engineered cysteines into FLAG-D2 dopamine receptor for disulfide crosslinking experiments, we reacted FLAG-D2 dopamine receptor in intact cells with copper phenanthroline (CuP), an oxidizing reagent that promotes the formation of disulfide bonds directly between cysteines (71,72). Reaction with CuP produced a new band of approx 133 kDa (Fig. 1A), which is approximately twice the size of monomer (11). The fraction of total density that was present in the approx 133-kDa band was plotted against increasing CuP concentrations (Fig. 1), providing half-maximal crosslinking at $60 \pm 10 \mu\text{M}$ CuP and maximal crosslinking of $80 \pm 14\%$ ($n = 3$).

The apparent mass of the crosslinked species was consistent with it being a homodimer of D2 dopamine receptor; however, because it was possible that it might represent D2 dopamine receptor crosslinked to another protein of similar size, the partners in the crosslinked species were definitively identified by co-immunoprecipitation of *myc*-D2 dopamine receptor stably co-expressed with FLAG-D2 dopamine receptor (11). These results established that the approx 133-kDa band is a D2 dopamine receptor homodimer that is disulfide crosslinked via one of the remaining endogenous cysteines. Mutation of Cys168^{4,58}, but not Cys56^{1,54}, Cys126^{3,44}, or Cys356^{6,47}, in TM4 to Ser completely prevented CuP-induced crosslinking (Fig. 2; ref. 11), dem-

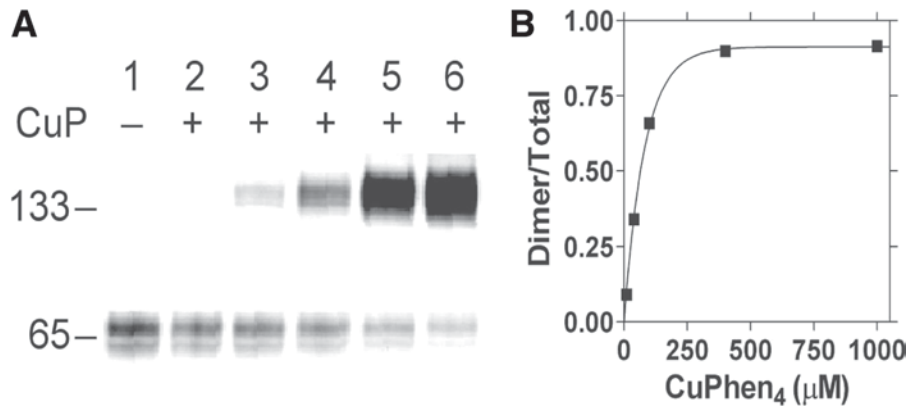


Fig.1. Crosslinking of D2 dopamine receptor to a homodimer by copper phenanthroline. (A) Treatment of FLAG–D2 dopamine receptor with 0, 10/40, 40/160, 100/400, 400/1600, 1000/4000 mM CuP (lanes 1–6, respectively). (B) Exponential association fit of dimer/total density plotted against CuP from panel a. The molecular masses of protein standards are given in kDa. Representative data from $n = 3$ experiments are shown. (Adapted from ref. 11.)

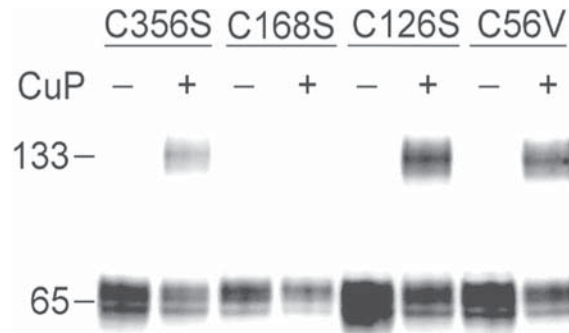


Fig. 2. Crosslinking of Cys mutants by 100/400 mM CuP in FLAG–D2 dopamine receptor. Representative data from $n = 3$ experiments are shown. (Adapted from ref. 11.)

onstrating that this Cys at the extracellular end of TM4 forms the CuP-induced disulfide crosslink at a symmetrical homodimer interface. However, mutation of Cys168^{4,58} to Ser does not prevent interaction at this D2 dopamine receptor dimer interface, based on the crosslinking of this construct observed when another residue at the same interface is simultaneously mutated to cysteine (unpublished observations).

Because crosslinking requires that only one of the two cysteines involved is modified initially by the reagent, and the derivatized cysteine then reacts by collision with the second unmodified cysteine, the rate of collision must be much faster than the rate of initial modification. This is consistent with the cysteines being very close initially. The very high fraction of receptor that can be crosslinked, the apparent specificity of the crosslinking (based on the appearance of a single homodimer band), and the lack of crosslinking of Cys56^{1.54} (which, based on the bovine rhodopsin structure, has a lipid accessibility similar to Cys168^{4.58}) all argue for the proximity of the TM4 cysteines in the native state. Therefore, in the membrane, D2 dopamine receptor, untreated with CuP, very likely exists as a homodimer, but this dimer does not survive detergent solubilization.

Our finding that the site of crosslinking in D2 dopamine receptor is Cys168^{4.58} at the extracellular end of TM4 is consistent with the hypothesis that TM4 forms a symmetrical dimer interface. Notably, the computational method we described earlier predicted C^{4.58} as a possible dimeric contact in D2 dopamine receptor, together with I^{4.48}, T^{4.55}, and L^{4.60}. To reduce the number of false-positives among the predicted correlated mutations and to reveal additional loci of interaction between monomeric interfaces as discussed in detail earlier, we recently built geometrically feasible configurations of D2 dopamine receptor homodimers (in preparation). The information available from these monomer-based models is currently guiding experiments that are serving to map the entire interface of D2 dopamine receptor dimerization, with the goal of understanding the role of the interface in ligand binding and receptor activation and of discovering mutations that disrupt the interface between monomers, which therefore interfere with receptor function. Because of the success in the application of our interdisciplinary approach to D2 dopamine receptors, we propose to use our methodology as a tool to provide new insights into the understanding of the structural basis and functional consequences of GPCR oligomerization.

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