D2 Dopamine Receptor Homodimerization Is Mediated by Multiple Sites of Interaction, Including an Intermolecular Interaction Involving Transmembrane Domain 4[†]

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ABSTRACT: In this study, we examined the mechanisms of intermolecular interaction involved in D2 dopamine receptor dimer formation to develop an understanding of the quaternary structure of G protein-coupled receptors. The potential role of two mechanisms was investigated: disulfide bridges and hydrophobic interactions between transmembrane domains. D2 dopamine receptor oligomers were unaffected by treatment with a reducing agent; however, oligomers of the D1 dopamine receptor dissociated following a similar treatment. This observation suggested that other forces such as hydrophobic interactions were more robust in the D2 receptor than in the D1 receptor in maintaining oligomerization. To elucidate which transmembrane domains were involved in the intermolecular hydrophobic interactions, truncation mutants were generated by successive deletion of transmembrane domains from amino and/or carboxyl portions of the D2 dopamine receptor. Immunoblot analyses revealed that all the fragments were well expressed but only fragments containing transmembrane domain 4 were able to self-associate, suggesting that critical areas for receptor dimerization resided within this transmembrane domain. Disruption of the helical structure of transmembrane domain 4 in a truncated receptor capable of forming dimers interfered with its ability to self-associate; however, a similar disruption of the transmembrane domain 4 helix structure in the full-length receptor did not significantly affect dimerization. These results indicated that there are other sites of interaction involved in D2 receptor oligomer assembly in addition to transmembrane domain 4.

Although the G protein-coupled receptor (GPCR)[1] superfamily is divided into divergent families whose sequences are not similar (*1*), these receptors are all characterized structurally by seven hydrophobic transmembrane (TM) domains that are connected by extracellular and intracellular loops. It had largely been thought that GPCRs existed as monomeric entities; however, it is now recognized that GPCRs form homo- and hetero-oligomers (for recent reviews, see refs *2* and *3*), and this recognition has resulted in a revision of the traditional models of GPCR structure and function. Dopamine receptors are members of the rhodopsin-like GPCR family (also termed family 1). In one of the earliest direct demonstrations of receptor−receptor interactions involving GPCRs, we showed that the D2 dopamine receptor forms sodium dodecyl sulfate (SDS) resistant homodimers (*4*). In subsequent studies, we have observed dimeric D2 receptors in human and rat brain (*5*) and have demonstrated that these receptors may also form higher-order homo-oligomers (*5*−*7*).

There is evidence that some dopaminergic ligands may have different affinities for dimeric receptors than for monomeric receptors (*6*) and that dimeric D2 dopamine receptors exhibit negative cooperativity, a property of receptors with interacting binding sites in which the binding of a ligand to one site modulates the binding of a second ligand to another site, for the antagonist raclopride (*8*). It has also been shown that that oligomerization of the D2 dopamine receptor occurred before it was transported to the cell membrane and that correctly formed oligomeric D2 dopamine receptor complexes appear to be required for cell surface expression (*7*).

Although the understanding of the functional significance of GPCR oligomers is improving, the structural basis of the intermolecular interactions involved is not completely understood. Significant progress has been made in the elucidation of some of the mechanisms of dimerization in family 3 GPCRs (*9*−*14*). For example, the recent crystallization of the extracellular amino termini of the mGluR1 metabotropic glutamate receptor dimers has demonstrated that this receptor is a covalently linked dimer associated with intermolecular

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[1] Abbreviations: GPCR, G protein-coupled receptor; TM, transmembrane; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; AT, amino terminus; CT, carboxyl terminus; ICL, intracellular loop.

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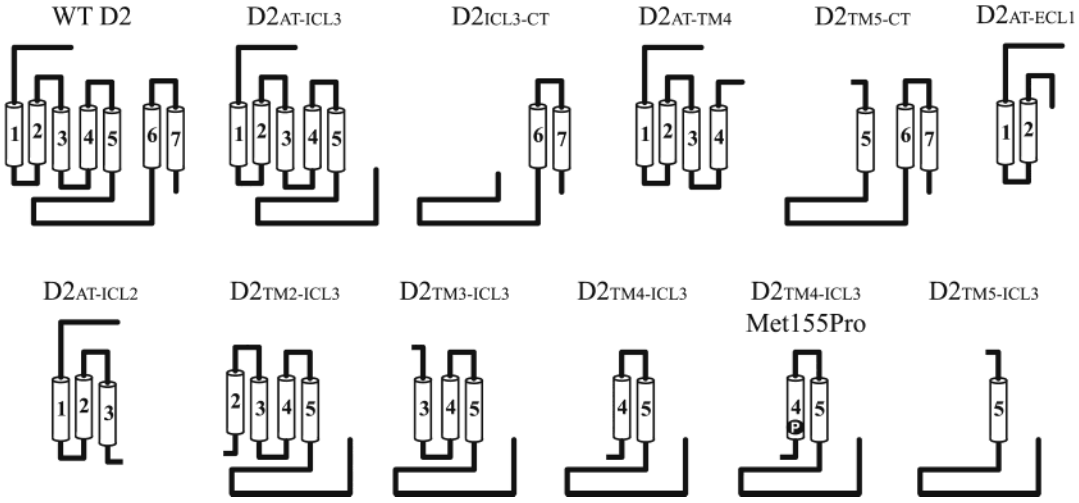

FIGURE 1: Schematic representation of the D2 dopamine receptor and truncation mutants. In the naming system for the truncations, AT is the amino terminus, CT the carboxyl terminus, ICL the intracellular loop, ECL the extracellular loop, and TM the transmembrane domain.

disulfide bridges (*9*). Because of the dissimilar structure, however, few specific parallels can be drawn for the rhodopsin-like family of GPCRs such as the D2 dopamine receptor.

Dissociation of receptor homodimers by a reducing agent has been shown for several rhodopsin-like GPCRs, including the δ- and κ-opioid receptors (*15*, *16*), the V2 vasopressin receptor (*17*), and the M3 muscarinic receptor (*18*). These observations suggest that disulfide bridges have a role in GPCR dimerization, but it is not clear if there are intermolecular covalent linkages. Intermolecular hydrophobic interactions between TM domains have also been proposed as a mechanism for GPCR dimerization (*7*, *17*), but the sites of interaction have not been precisely defined.

In the study presented here, to learn more about the receptor−receptor associations in GPCRs, we examined disulfide bonds potentially involved in receptor dimerization and demonstrated that disruption of disulfide linkages did not significantly dissociate D2 dopamine receptor dimers. Upon examination of intermolecular TM domain interactions, it was shown that these interactions and not covalent linkages predominated in dimer formation. Subsequently, since it has been shown that truncated forms of receptors retain helix−helix interaction motifs involved in oligomerization of the native protein (*19*, *20*), we used truncation mutants to determine that TM domain 4 was an important site of intermolecular interaction mediating D2 dopamine receptor dimerization. This identification of a specific region that is critical for receptor dimer formation represents a significant advance in the understanding of the quaternary structure of the D2 dopamine receptor.

EXPERIMENTAL PROCEDURES

Construction of the Expression Vectors for the D2 Dopamine Receptor and Truncation Mutants. cDNA encoding the short isoform of the D2 dopamine receptor (kindly provided by O. Civelli, University of California, Irvine, CA) was used as the template in the polymerase chain reaction (PCR) construction of the cDNA constructs. The start and end positions of the truncations relative to the amino acid sequence of wild-type D2 dopamine receptor were as follows: 1−373 for $D2_{AT-ICL3}$, 1−181 for $D2_{AT-TM4}$, 1−106 for $D2_{AT-ECL1}$, 1−149 for $D2_{AT-ICL2}$, 62−373 for $D2_{TM2-ICL3}$, 99−373 for $D2_{TM3-ICL3}$, 141−373 for $D2_{TM4-ICL3}$, 178−373 for $D2_{TM5-ICL3}$, 178−443 for $D2_{TM5-CT}$, and 212−443 for $D2_{ICL3-CT}$. Schematic representations of the mutants are shown in Figure 1. Flag epitope (DYKDDDDK) tagging at the N-terminus of the D2 dopamine receptor and C9 epitope (TETSQVAPA) tagging at the C-terminus of $D2_{AT-TM4}$ were also achieved by PCR. The products were inserted into the pcDNA3 mammalian expression vector.

Construction of the Expression Vectors for the D2 Dopamine Receptor N-Linked Glycosylation Mutants. Using oligonucleotides designed to anneal to DNA encoding the N-terminal region of the D2 dopamine receptor but encoding Asn to Ala mutations at amino acid positions 5, 17, and 23, D2 receptor mutants lacking one to three N-linked glycosylation sites were generated by PCR. Generally, for GPCRs, no effect on receptor function has been observed as a result of removal of putative glycosylation sites; however, a reduced expression level or impaired plasma membrane targeting has been observed for some receptors (*21*, *22*). Therefore, the expression level of the D2 mutant receptors lacking glycosylation sites was determined by one-point, saturating concentration, binding experiments using [^{3}H]nemonapride.

COS-7 Cell Culture. COS-7 cells were maintained as monolayer cultures at 37 °C in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics. The cells were transiently transfected using lipofectamine reagent (Life Technologies). Membranes were prepared 48 h post-transfection.

Sf9 Cell Culture and D2 Dopamine Receptor Expression. Details of the construction of the recombinant baculovirus encoding the human D2 dopamine receptor cDNA, the c-myc epitope-tagged human D1 dopamine receptor, the c-myc-tagged human D3 dopamine receptor, and the c-myc-tagged human 5-HT_{1D} serotonin receptor have been reported previously (*4*, *23*−*25*). Sf9 cells were maintained at 27 °C in Grace's Insect Medium supplemented with 10% (v/v) fetal bovine serum, 0.5× antibiotic-antimycotic, and 1% (v/v) Pluronic F-68, a surfactant. The cells were grown either as monolayer cultures in T flasks or as suspension cultures in spinner flasks. Suspension cultures were infected with

baculovirus when the cell density was between 1×10^6 and 3×10^6 cells/mL with a multiplicity of infection of approximately 5. Membranes were prepared from the cells 72 h post-infection.

Membrane Preparation. Cells were washed with phosphate-buffered saline, resuspended in hypotonic lysis buffer with protease inhibitors [5 mM Tris-HCl, 2 mM EDTA, 5 μg/mL leupeptin, 10 μg/mL benzamide, and 5 μg/mL soybean trypsin inhibitor (pH 7.4)], and homogenized with a Brinkman Polytron apparatus. The homogenate was centrifuged to pellet unbroken cells and nuclei, and the supernatant was collected. The supernatant was centrifuged at 40000*g* for 20 min to isolate a membrane fraction enriched with, but not exclusively consisting of, plasma membrane (*26*), and the resulting pellet (P2 membranes) was washed and resuspended in lysis buffer.

Antibodies. The rabbit polyclonal antibody AL-26 (a gift of M. Brann, Acadia Pharmaceuticals, San Diego, CA) was raised against a GST fusion protein with a portion of the third intracellular loop of the human D2 dopamine receptor (amino acids 220−340). The specificity of this antibody for the D2 dopamine receptor with no cross reactivity to other GPCRs has been reported elsewhere (*27*). The B-10 anti-D2 dopamine receptor antibody which was directed against the first 50 amino acids of the human D2 dopamine receptor (Santa Cruz Biotechnology, Sanata Cruz, CA) was used to probe for receptor truncation mutants lacking the third intracellular loop. The M5 anti-Flag antibody (Sigma-Aldrich, St. Louis, MO) was used for detection of the Flag epitope. The 1D4 antibody recognizing the C9 epitope was obtained from the National Cell Culture Center.

SDS−PAGE and Immunoblotting. The protein samples were subjected to reducing or nonreducing SDS−PAGE using 10 or 12% precast acrylamide gels (Novex/Invitrogen, Carlsbad, CA) and transferred to nitrocellulose using a semidry transfer apparatus. The 2× SDS sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, and 0.005% bromophenol blue] contained 5% β-mercaptoethanol when electrophoresis was performed under reducing conditions. The nitrocellulose was blocked using 10% (w/v) skim milk powder in Tris-buffered saline (TBS) and then incubated in 1% skim milk powder TBS containing the primary antibody. Visualization was by alkaline phosphatase detection or chemiluminescence detection.

Treatment of D2 Dopamine Receptor-Expressing Membranes with a Reducing Agent. Membranes were pelleted and resuspended in a 0.1 M Tris-HCl (pH 7.5) solution. An aliquot of a freshly prepared 1 M dithiothreitol (DTT) solution was mixed with the membrane suspension for a final DTT concentration of 50 mM. The mixture was allowed to incubate for 30 min at room temperature, then mixed with 2× SDS sample buffer, and subjected to electrophoresis.

RESULTS

Analysis of the Oligomeric Forms of the D2 Dopamine Receptor. When analyzed by immunoblotting, D2 dopamine receptors expressed in COS7 cells were detected as bands with apparent molecular masses of ∼50, ∼100, and ∼200 kDa (Figure 2, lane 1). Since the molecular mass predicted by its amino acid sequence is ∼45 kDa and GPCRs are glycoproteins, i.e., subject to N-linked glycosylation which

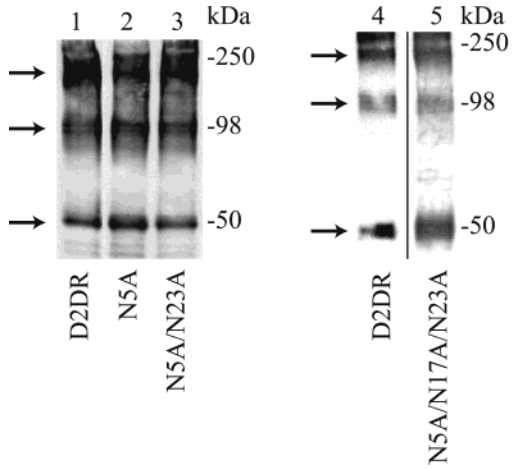

FIGURE 2: Immunoblot analysis of D2 dopamine receptor glycosylation mutants. Membranes from COS7 cells expressing the Flag-D2 dopamine receptor (lanes 1 and 4), Flag-D2-N5A (lane 2), Flag-D2-N5A/N23A (lane 3), and Flag-D2-N5A/N17A/N23A (lane 5) were subjected to SDS−PAGE and immunoblotted with M5 (lanes 1−3) or AL-26 (lanes 4 and 5) antibody. The immunoblots in lanes 4 and 5 were exposed to film for ∼5 and 60 s, respectively. Radioligand-detected receptor density estimates for the membranes were as follows: 1.2 ± 0.3 pmol/mg of protein for the Flag-D2 dopamine receptor, 1.6 ± 0.3 pmol/mg of protein for Flag-D2-N5A, 1.8 ± 0.4 pmol/mg of protein for Flag-D2-N5A/N23A, and 0.65 ± 0.16 pmol/mg of protein for Flag-D2-N5A/N17A/N23A. Arrows denote receptor monomers, dimers, and higher-order oligomers. For each lane, 25 μg of protein was used.

can greatly increase the apparent molecular mass of receptor monomers, we examined the effect of N-linked glycosylation on the molecular mass of the D2 dopamine receptor. The D2 dopamine receptor has only three putative N-linked glycosylation sites, all of which are in the amino terminus. To test the extent of glycosylation, we generated substitution mutants lacking one, two, or all three of the N-linked glycosylation sites. When N5A, N5A/N23A, or N5A/N17A/N23A D2 dopamine receptors were expressed in COS7 cells and immunoblotted (Figure 2, lanes 2, 3, and 5), the immunodetected bands were the same as observed for the wild-type receptor. These observations suggest that, in COS7 cells, the apparent molecular mass of the D2 dopamine receptor is not significantly affected by N-linked glycosylation or that these receptors are not N-glycosylated when expressed in COS7 cells. Therefore, we concluded that the ∼100 and ∼200 kDa bands do not represent glycosylated forms of D2 dopamine receptors and that the ∼50 and ∼100 kDa species were the monomeric and dimeric forms of the receptor, respectively.

Intermolecular Interactions Elucidated Using Truncation Mutants. It has been shown that peptides and receptor truncation mutants can mimic TM segments of membrane proteins (*19*, *20*, *28*) and retain helix−helix interactions involved in oligomerization of the protein (*19*, *20*). We have also shown that truncated receptor fragments, when co-expressed, can reconstitute into receptors with ligand binding properties and signaling identical to those of the full-length receptors (*7*). Furthermore, we have demonstrated by circular dichroism spectroscopy that TM peptides maintain α-helical structure.[2] Therefore, to analyze the intermolecular TM domain interactions mediating dimerization of the D2

[2] Unpublished data.

dopamine receptor, we employed a strategy using truncation mutants designed with deletions of entire TM domains. Initially, we generated two truncated forms: $D2_{AT-ICL3}$ which consisted of the first 373 amino acids, including only the first five TM domains, and $D2_{ICL3-CT}$ which contained the terminal 203 amino acids (212−414), including only TM domains 6 and 7. When these truncation mutants were expressed alone, both were detected in the P2 membrane fraction, and when coexpressed, the two receptor fragments reconstituted a receptor capable of agonist-mediated adenylyl cyclase inhibition and with pharmacology similar to that of the wild-type D2 dopamine receptor (*7*), indicating that these fragments were structurally intact. When analyzed by immunoblotting under reducing conditions, $D2_{AT-ICL3}$ was visualized as monomers (∼44 kDa) and dimers (∼90 kDa) (Figure 3, lane 1), whereas only monomeric $D2_{ICL3-CT}$ (∼34 kDa) was immunodetected (Figure 3, lane 7). On the basis of this observation, we hypothesized that one or more of the sites of interaction involved in D2 dopamine oligomerization were preserved in $D2_{AT-ICL3}$ and absent in $D2_{ICL3-CT}$.

To investigate this hypothesis further, we generated several other D2 dopamine receptor fragments. Serial N-terminal truncations of $D2_{AT-ICL3}$ designed to successively delete TM domains were expressed in COS7 cells, and all were detected in the P2 membrane fraction by immunoblot analysis. Under reducing conditions, immunoreactive bands corresponding to monomers and dimers were observed for $D2_{TM2-ICL3}$ (Figure 3, lane 2), $D2_{TM3-ICL3}$ (lane 3), and $D2_{TM4-ICL3}$ (lane 4), but only monomers were detected for $D2_{TM5-ICL3}$ (lane 5). A truncation mutant with a deletion of the first four TM domains, $D2_{TM5-CT}$, was also only visualized as monomers (lane 6). The approximate molecular sizes for the immunodetected monomeric fragments were as follows: ∼40 kDa for $D2_{TM2-ICL3}$, ∼38 kDa for $D2_{TM3-ICL3}$, ∼35 kDa for $D2_{TM4-ICL3}$, ∼34 kDa for $D2_{TM5-ICL3}$, and ∼36 kDa for $D2_{TM5-CT}$. These truncation mutants lack the N-terminus of the wild-type D2 dopamine receptor and therefore do not have any N-linked glycosylation sites. A further truncation of the C-terminus of $D2_{AT-ICL3}$ termed $D2_{AT-TM4}$ was visualized as dimers (Figure 3, lane 8), but only monomers of the additionally truncated $D2_{AT-ICL2}$ (lane 9) and $D2_{AT-ECL1}$ (lane 10) were observed. The $D2_{AT-ECL1}$ mutant was seen as a doublet band, suggesting that this truncation may have multiple conformations. Monomers of $D2_{AT-TM4}$ were not detected. Since the structural basis of D2 dopamine receptor dimerization appears to be intact in the $D2_{TM4-ICL3}$ receptor fragment and in $D2_{AT-TM4}$ but absent in $D2_{AT-ICL2}$, $D2_{ICL3-CT}$, $D2_{TM5-ICL3}$, and $D2_{TM5-CT}$, it can be inferred that a region within TM domain 4 contained a minimal structural requirement for dimerization.

Disruption of the TM 4 Interface. We attempted to determine if disturbance of the helical structure of TM 4 would disrupt the dimer interface between D2 dopamine receptors. The introduction of a proline residue into a TM α-helix, particularly within regions near the membrane interface, has been shown to break the helix structure (*29*). Therefore, we substituted a proline in the $D2_{TM4-ICL3}$ receptor truncation at a position corresponding to the amino acid predicted to be five residues from the intracellular loop in the full-length receptor (Met155). When examined by immunoblotting, the Met155Pro $D2_{TM4-ICL3}$ mutant was visualized primarily as monomers, with no dimers or higher-order oligomers detected (Figure 4A). Notably, the immunodetected band for Met155Pro $D2_{TM4-ICL3}$ migrated at an apparent molecular mass slightly higher than that of monomeric $D2_{TM4-ICL3}$, suggesting that the conformation of the proline mutant was significantly altered. When the analogous mutation was generated in the full-length D2 dopamine receptor (Met155Pro-D2DR) and subjected to immunoblot analysis, no significant differences in the oligomerization of

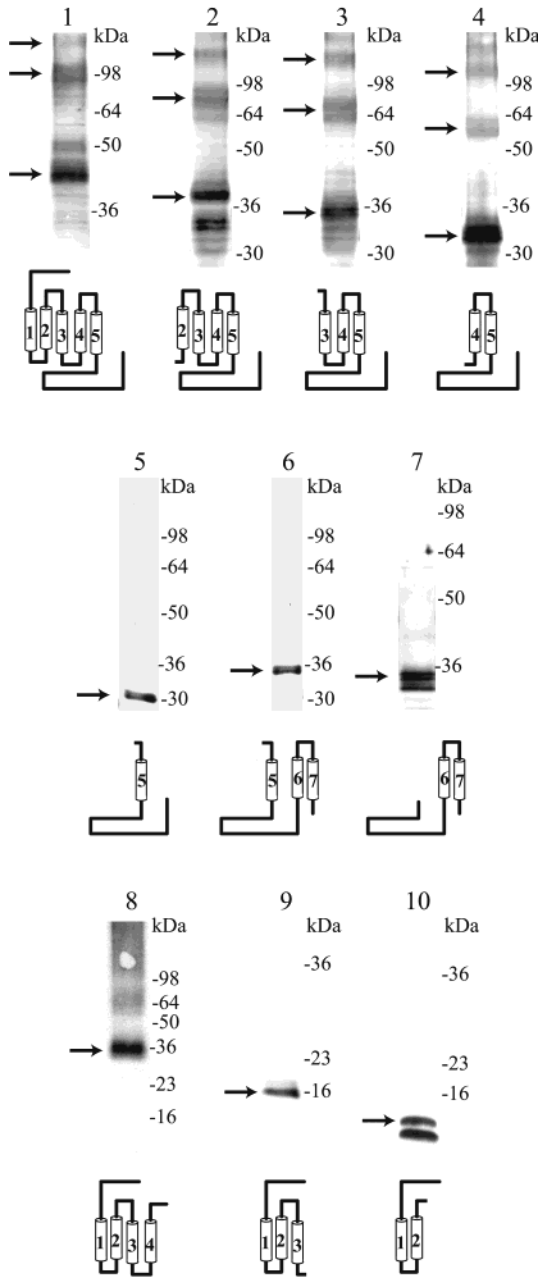

FIGURE 3: Immunoblot analysis of D2 dopamine receptor truncation mutants. Membranes from COS7 cells transiently transfected with cDNA encoding $D2_{AT-ICL3}$ (lane 1), $D2_{TM2-ICL3}$ (lane 2), $D2_{TM3-ICL3}$ (lane 3), $D2_{TM4-ICL3}$ (lane 4), $D2_{TM5-ICL3}$ (lane 5), $D2_{TM5-CT}$ (lane 6), $D2_{TM6-CT}$ (lane 7), $D2_{AT-ECL2}$ (lane 8), $D2_{AT-ICL2}$ (lane 9), or $D2_{AT-ECL1}$ (lane 10) were subjected to SDS−PAGE under reducing conditions and immunoblotted. AL-26 (lanes 1−7), anti-C9 antibody (lane 8), or B-10 anti-D2DR antibody (lanes 9 and 10) was used for immunodetection. The immunoblots shown in lanes 1−7 are from the same gel, and this immunoblot is representative of several replicates ($n = 4$). Arrows denote monomers, dimers, and higher-order oligomers. For each lane, 25 μg of protein was used.

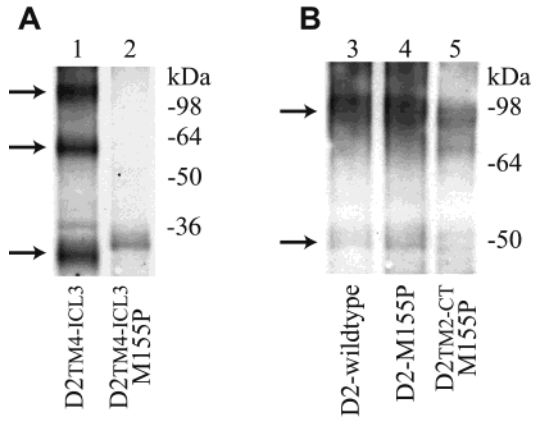

FIGURE 4: Immunoblot analysis of TM domain 4 substitution mutant $D2_{TM4-ICL3}$ and of the D2 dopamine receptor. (A) Membranes from COS7 cells expressing the $D2_{TM4-ICL3}$ truncation mutant (lane 1) or $D2_{TM4-ICL3}$-M155P (lane 2) were subjected to SDS−PAGE and immunoblotted with AL-26 antibody. (B) Membranes from COS7 cells expressing the wild-type D2 dopamine receptor (lane 3), the D2-M155P point mutant receptor (lane 4), or the M155P mutant of the $D2_{TM2-CT}$ truncation mutant (lane 5) were subjected to SDS−PAGE and immunoblotted with AL-26 antibody. Arrows denote receptor monomers and dimers. For each lane, 25 μg of protein was used.

the mutant could be observed when compared to the wild-type receptor (Figure 4B). Radioligand binding studies of Met155Pro-D2DR showed that the mutant was able to bind dopaminergic ligands; however, the binding affinities were significantly decreased. The K_d values of [^{3}H]nemonapride binding for the wild-type D2 receptor and the Met155Pro mutant were 327 ± 83 and 2827 ± 608 pM, respectively, and the K_d values for [^{3}H]spiperone binding were 651 ± 245 and 2280 ± 394 pM for the wild-type D2 receptor and the Met155Pro mutant, respectively. These observations indicated that there were additional intermolecular interactions in the full-length D2 dopamine receptor in addition to the TM 4 interface.

It has been demonstrated recently that a mutant of the yeast α-mating factor receptor lacking the amino-terminal domain and TM 1 did not self-associate into homodimers (*30*). Although we have shown with the $D2_{TM2-ICL3}$ mutant (Figure 3) that the amino-terminal domain and TM 1 are not required for D2 dopamine receptor dimerization, it was possible that a site of intermolecular interaction was located within these regions. We hypothesized that the absence of the amino-terminal domain and TM 1 in the D2 dopamine receptor in which the TM 4 interface has been disturbed may affect dimerization. To test this hypothesis, a truncation of the Met155Pro D2 dopamine receptor mutant (Met155Pro-$D2_{TM2-CT}$) was created. Immunoblotting of Met155Pro-$D2_{TM2-CT}$ revealed that this mutant was able to form dimers (Figure 4B, lane 5), suggesting either that the amino-terminal domain and TM 1 do not contain a site of intermolecular interaction for the D2 dopamine receptor or that there are more than two dimerization interfaces.

Differential Sensitivity of GPCR Dimers to a Reducing Agent. To determine whether disulfide bridges also had a role in D2 dopamine receptor dimerization, membranes from D2 dopamine receptor-expressing cells were treated with the reducing agent DTT. Untreated membranes, immunoblotted under nonreducing conditions, were compared to DTT-treated membranes, immunoblotted under reducing conditions, and little dissociation of receptor dimers by DTT was detected (lanes 1 and 2 of Figure 5A and lanes 1 and 2 of Figure 5B). Furthermore, no dissociation of dimers was observed when D2 dopamine receptor-expressing whole cells were subjected to DTT treatment (data not shown). Similar results were obtained when the D3 dopamine receptor, a closely related member of the D2 subfamily of dopamine receptors, was treated with a reducing agent (lanes 5 and 6 of Figure 5A). However, when membranes from cells expressing the D1 dopamine receptor (lanes 3 and 4 of Figure 5A and lanes 3 and 4 of Figure 5B) or the serotonin 5-HT1D receptor (lanes 7 and 8 of Figure 5) were subjected to DTT, we observed that oligomers visualized by immunoblot analysis exhibited a greater sensitivity to dissociation by a reducing agent. Notably, a complete disruption of all oligomers to monomers could not be achieved in any of the receptors, indicating that dimerization of these receptors was mediated by additional factors such as TM domain interactions.

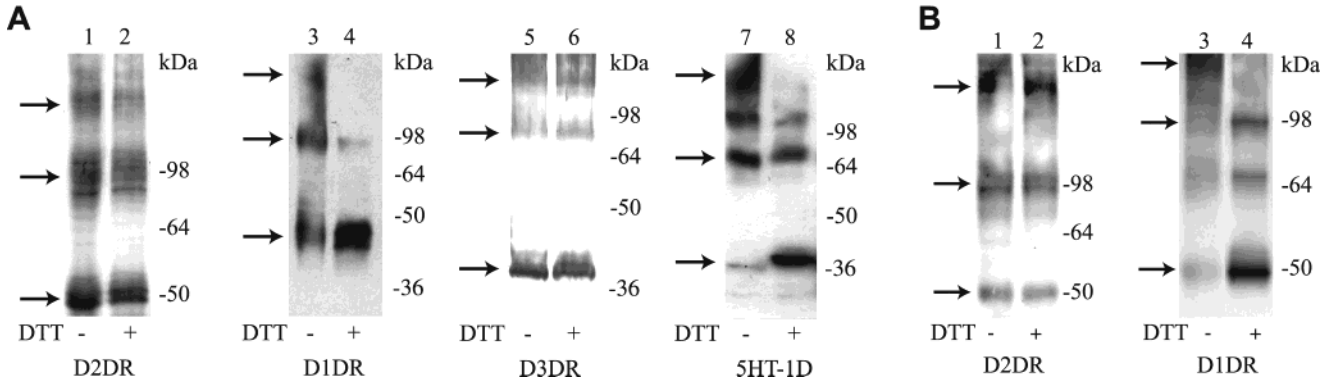

FIGURE 5: Effect of a reducing agent on the G protein-coupled receptor oligomers. (A) Membranes from Sf9 cells expressing the D2 dopamine receptor (lanes 1 and 2), the c-myc epitope-tagged D1 dopamine receptor (lanes 3 and 4), the c-myc epitope-tagged D3 dopamine receptor (lanes 5 and 6), or the c-myc epitope-tagged 5-HT_{1D} serotonin receptor (lanes 7 and 8) were treated with 50 mM DTT for 30 min (lanes 2, 4, 6, and 8). Membranes in lanes 1, 3, 5, and 7 were subjected to electrophoresis under nonreducing conditions. AL-26 (lanes 1 and 2) or 9E10 anti-c-myc antibodies (lanes 3−8) were used for immunodetection. (B) Membranes from COS7 cells expressing the D2 dopamine receptor (lanes 1 and 2) and the HA epitope-tagged D1 dopamine receptor (lanes 3 and 4) were treated with DTT as described above (lanes 2 and 4). Membranes in lanes 1 and 3 were subjected to electrophoresis under nonreducing conditions. AL-26 (lanes 1 and 2) or 3F10 anti-HA antibodies (lanes 3 and 4) were used for immunodetection. Arrows denote monomers, dimers, and higher-order oligomers. For each lane, 25 μg of protein was used.

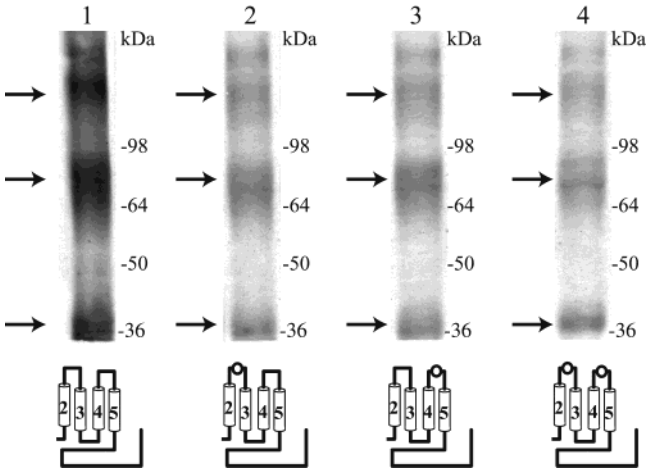

FIGURE 6: Immunoblot analysis of cysteine mutations in the extracellular loops of the $D2_{TM2-ICL3}$ truncation mutant. Membranes from COS7 cells expressing $D2_{TM2-ICL3}$ (lane 1), $D2_{TM2-ICL3}$-C107A (lane 2), $D2_{TM2-ICL3}$-C182A (lane 3), or $D2_{TM2-ICL3}$-C107A/C182A (lane 4) were immunoblotted using the AL-26 antibody. Arrows denote monomers, dimers, and higher-order oligomers. For each lane, 25 μg of protein was used. The positions of the mutated cysteine residues are for the wild-type D2 dopamine receptor.

Mutation of Cysteine Residues in Extracellular Loops 1 and 2. Since it is plausible that the lack of sensitivity of D2 dopamine receptor oligomers to the reducing agent may be the result of disulfide bridges being less accessible than in other GPCRs due to the TM domain architecture, we employed a mutagenesis strategy using the $D2_{TM2-ICL3}$ construct. There are several highly conserved cysteine residues in rhodopsin-like GPCRs. Among these residues are a cysteine in the first extracellular loop and another in the second extracellular loop that are present in almost all family 1 receptors, including the D2 dopamine receptor. It has been shown that, when one or both of these conserved cysteines were mutated in the M3 muscarinic receptor, the proportion of dimers visualized by immunoblotting was markedly decreased (*31*). To investigate the role of the cysteine residues in extracellular loops 1 and 2 of the D2 dopamine receptor in oligomerization, Cys → Ala mutations in the extracellular loops of the $D2_{TM2-ICL3}$ fragment were examined. Immunoblot analysis of membranes from cells expressing single or double extracellular cysteine mutations of $D2_{TM2-ICL3}$ revealed that oligomerization of the fragments was unaffected, but the expression levels of the three mutant fragments were not as high as that of the "wild-type" fragment (Figure 6). This observation indicated that the two cysteine residues in extracellular loops 1 and 2 of the D2 dopamine receptor did not have a detectable role in dimerization.

Differential Sensitivity of D2 Dopamine Receptor Fragments to a Reducing Agent. We postulated that disulfide bridges in $D2_{TM2-ICL3}$ or $D2_{TM4-ICL3}$ may be more accessible to a reducing agent than in the wild-type receptor, and we investigated the effects of DTT treatment on these truncation mutants. When $D2_{TM2-ICL3}$ and $D2_{TM4-ICL3}$ were analyzed by nonreducing SDS−PAGE and compared to the immunoblots obtained under reducing conditions, no detectable alteration in the ratio of monomers to dimers was detected (data not shown). Treatment of membranes expressing $D2_{TM2-ICL3}$ and $D2_{TM4-ICL3}$ with DTT prior to reducing SDS−PAGE resulted in an increased level of immunodetection of monomers, but a significant reduction in the number of receptor fragment dimers was not observed (Figure 7).

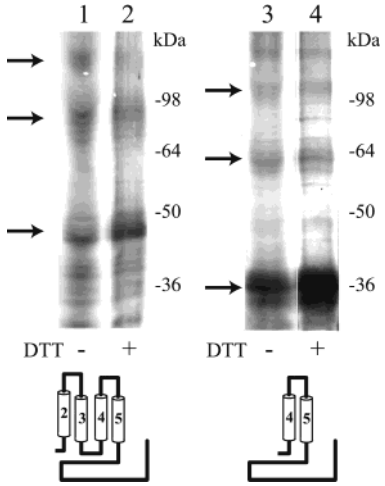

FIGURE 7: Effect of a reducing agent on D2 dopamine receptor truncation mutants. Membranes from COS7 cells expressing $D2_{TM2-ICL3}$ (lanes 1 and 2) or $D2_{TM4-ICL3}$ (lanes 3 and 4) were treated with 50 mM DTT for 30 min. Membranes in lanes 1 and 3 were subjected to electrophoresis under nonreducing conditions. Arrows denote monomers, dimers, and higher-order oligomers. For each lane, 25 μg of protein was used.

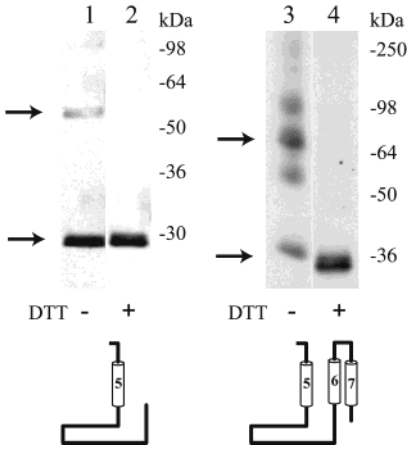

FIGURE 8: Effect of a reducing agent on D2 dopamine receptor truncation mutants lacking TM domain 4. Membranes from COS7 cells expressing $D2_{TM5-ICL3}$ (lanes 1 and 2) or $D2_{TM5-CT}$ (lanes 3 and 4) were treated with 50 mM DTT for 30 min. Membranes in lanes 1 and 3 were subjected to electrophoresis under nonreducing conditions. Arrows denote monomers, dimers, and higher-order oligomers. For each lane, 25 μg of protein was used.

Interestingly, when membranes from cells expressing $D2_{ICL3-CT}$ or $D2_{TM5-ICL3}$ were subjected to nonreducing immunoblot analysis, these two receptor fragments were visualized as monomers and dimers (Figure 8, lanes 1 and 3). Since nonspecific disulfide bond formation may occur during cell lysis and membrane preparation, the experiment was repeated using membranes prepared with lysis buffer containing 100 mM iodoacetamide, a sulfhydryl alkylating agent. The addition of the alkylating agent to the membrane preparation did not alter the fragment dimers visualized by nonreducing immunoblot analysis (data not shown). These observations suggested that disulfide linkages that are important for dimerization may be present in the full-length D2 dopamine receptor, but reduction of these bonds does

not lead to dissociation of the oligomers due to the dominant interactions involving TM domain 4.

DISCUSSION

In this study, we have investigated intermolecular TM domain interactions in the D2 dopamine receptor by employing a methodical strategy of expressing a series of truncation mutants with successively deleted TM domains. With this strategy, we have identified TM domain 4 as being a site of interaction in receptor dimerization. Like the full-length receptor, D2 receptor truncation mutants incorporating TM domains 4 and 5 (e.g., $D2_{TM4-ICL3}$) or consisting of TM domains 1−4 ($D2_{AT-TM4}$) were able to form dimers. However, truncation mutants lacking TM 4 such as the mutant consisting of TM domains 1−3 ($D2_{AT-ICL2}$) or the mutant composed of TM domains 5−7 ($D2_{TM5-CT}$) were visualized only as monomers. Taken together, these results indicated that TM 4 represents a dimerization interface and that, in this interface, TM 4 from one receptor interacts with TM 4 from its dimerization "partner". In confirmation of this determination, the disruption of the helical structure of TM 4 by the introduction of a proline residue in the truncation mutant composed of TM domains 4 and 5 ($D2_{TM4-ICL3}$) prevented it from forming dimers. Interestingly, however, the same mutation in the full-length receptor and in the $D2_{TM2-CT}$ truncation mutant had no detectable effect on dimer formation. This observation pointed to the presence of one or more additional intermolecular interactions in the full-length receptor that were absent or weaker in the $D2_{TM4-ICL3}$ truncation mutant. Therefore, although TM domain 4 contains a critical structural requirement for D2 receptor dimerization, there are additional interfaces participating in the dimerization process.

The determination of TM domain 4 as a site of intermolecular contact is consistent with the findings of a recent report which has shown that, in D2 dopamine receptor homodimers, the cysteine in TM4 at position 168 was adjacent and close to the same cysteine in its dimerization partner (*32*). In that study, D2 receptors were cross-linked at these two cysteine residues by copper phenanthroline and therefore indicated that the two TM 4 domains (or symmetrical portions thereof) were aligned closely to each other in a receptor homodimer. In our study, we have reached similar conclusions using an entirely different approach not employing chemical cross-linking and have shown an intermolecular association involving TM 4 in D2 dopamine receptor homodimers.

The precise nature of the interfaces other than TM domain 4 is not clear. A study on the V2 vasopressin receptor has suggested that a region or regions within TM domains 1−3 from one receptor may interact with TM domains 1−3 from another (*17*). However, the $D2_{AT-ICL2}$ fragment of the D2 receptor did not self-associate. Computational modeling of adrenergic receptors has suggested several possible sites of interaction between TM domains in receptor dimerization (*33*); however, this model is largely predicated on the idea that GPCRs participate in a process known as domain swapping (*34*), a finding called into question by recent experimental evidence (*35*, *36*). It is likely that GPCRs form "contact" dimers similar to that discussed by Schulz and coworkers where intact receptor monomers associate with each other (*36*).

Receptor fragments have been used in the past to investigate both the function and structure of GPCRs. Reconstitution of receptor fragments by coexpression into functional receptors has been shown for several GPCRs (*37*−*39*), including the D2 dopamine receptor (*7*) and for the related bacteriorhodopsin (*40*), demonstrating that receptor fragments can be folded, transported, and processed in a manner analogous to that of the full-length receptor. We and others have exploited this feature of receptor fragments and coexpressed fragments with the full-length receptors in an effort to understand GPCR oligomerization (*7*, *17*, *36*, *41*, *42*), but the study presented here is somewhat distinct because, unlike many of the previous studies, it examined receptor fragments in the P2 membrane fraction (not whole cell lysates) that were expressed alone and not coexpressed with other portions of the receptor from which it was derived.

It is possible that different receptor subtypes may use completely different dimerization interfaces to associate; however, given how well family 1 GPCRs map to the crystal structure of rhodopsin (*43*−*45*), it seems unlikely that drastic differences in quaternary structure would be seen between receptors in this family. Interestingly, TM domain 4 is the least conserved among family 1 GPCRs (*46*), and if TM domain 4 is a site of interaction in all rhodopsin-like GPCRs, this lack of homology could be a means of preventing undesirable associations between closely related receptor subtypes and preserve specificity.

Modeling of the D2 dopamine receptor to the crystal structure of bovine rhodopsin has predicted a high degree of overall structural homology (*47*). Very notably, two-dimensional crystal structures of squid rhodopsin have shown that these receptors are arranged in "ordered lattices" and that, in these lattices, the TM 4 helices of neighboring receptors are aligned adjacent to each other (*48*). The crystal structure of the squid rhodopsin lattice also confirmed our experimental data which showed that multiple sites of intermolecular contact are possible between TM domains of neighboring receptors as there appear to be several regions for potential intermolecular contacts. The authors of the study note that there is a docking of helix 8 (a non-membrane-spanning helix between TM 7 and the carboxyl terminus) with the loop between helices 5 and 6 (the third intracellular loop). However, our experiments with the truncation mutants in which the third intracellular loop and helix 8 are intact ($D2_{ICL3-CT}$ and $D2_{TM5-CT}$) suggest that if such a docking mechanism exists in the D2 dopamine receptor, the interaction is disrupted during SDS electrophoresis since these fragments were visualized as monomers. Nevertheless, the docking of helix 8 with the third intracellular loop is an intriguing idea when considered with our findings since such an interaction would allow homodimeric receptors symmetrically associated at TM domain 4 to link together to form higher-order oligomers. This hypothetical structural model for higher-order oligomers is even more intriguing in light of the recent atomic force microscopy images of oligomeric rhodopsin (*49*) which shows rhodopsin molecules arranged as a concatenation of dimers.

In this study, we also examined the role of disulfide bonds in D2 dopamine receptor dimerization. Dimers of the full-length D2 dopamine receptor and of truncation mutants containing TM domain 4 were resistant to dissociation by a reducing agent unlike oligomers of other, closely related

receptors. $D2_{ICL3-CT}$ and $D2_{TM5-ICL3}$ truncation mutants lacking TM domain 4 formed DTT-sensitive dimers, indicating that disulfide bridges important for dimerization may be present in the D2 dopamine receptor, but that reduction of these bonds does not lead to dissociation of the oligomers possibly because of the robust hydrophobic interactions involving TM domain 4.

The reduction of disulfide bonds resulting in the dissociation of receptor dimers and higher-order oligomers has been shown for several rhodopsin-like GPCRs, but it is not clear if this is the result of disruption of intermolecular disulfide bridges. It is plausible that disruption of an intramolecular linkage could result in a structural change which secondarily disrupts the dimer interface. For example, oligomers of the erythrocyte glucose transporter, GLUT1, are sensitive to dissociation by a reducing agent, but were shown to have no covalent intermolecular associations between the subunits (*50*). An intramolecular disulfide bridge in the extracellular domain of the GLUT1 subunits was shown to promote tetramerization likely by preserving the architecture of the monomer (*51*).

In conclusion, we have determined using a systematic and sequential deletion of entire TM domains that TM domain 4 contains a dimerization interface for the D2 dopamine receptor and that the two TM 4 domains in the receptor dimer interact with each other. We have also shown that there are additional interfaces in the full-length receptor that are involved in dimerization. Further, we have demonstrated that disulfide linkages within the D2 receptor may be important for dimerization but the intermolecular interactions between TM domains predominate. Our elucidation of the interface for D2 dopamine receptor dimers may yield clues to the principles governing the dimerization of all rhodopsin-like GPCRs. The realization that GPCRs form dimers has resulted in a re-evaluation of the structure−function relationship in this important class of receptors. New models are emerging which incorporate the significant progress that has been made in elucidating the role of oligomerization in receptor function; however, the manner in which GPCRs, particularly those in the rhodopsin-like family, form oligomers has not been thoroughly investigated. The findings of this report begin to elucidate some of the quaternary structure of GPCRs.

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