Inhibition of Cell Surface Expression by Mutant Receptors Demonstrates that D2 Dopamine Receptors Exist as Oligomers in the Cell

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ABSTRACT

Numerous mutant G protein-coupled receptors with diminished or no function have been described that are naturally occurring or that are the product of gene manipulation. It has largely been assumed that receptor mutants do not affect the function of the wild-type receptor; however, the occurrence of G protein-coupled receptor dimerization suggests the possibility that an intermolecular interaction between mutant and wild-type receptors can occur. We have shown previously that the D2 dopamine receptor (D2DR) exists as dimers in cell lines and brain tissue. In this study, we demonstrated that mutant D2DR can modulate the function of the wild-type D2DR. While attempting to elucidate the structure of the D2DR dimer, we demonstrated that nonfunctional D2DR substitution and truncation mutants antagonized wild-type D2DR function. Furthermore, from analyses of this interaction between the receptor mutants and the D2DR, using photoaffinity labeling, we provide evidence that the D2DR is oligomeric in the cell.

Dopamine receptors are G protein-coupled receptors (GPCRs) and have a topological motif of seven hydrophobic α-helical domains that span the lipid bilayer and are connected by extracellular and intracellular loops. Until recently, mechanisms of GPCR ligand binding and signal transduction have been modeled with the assumption that only monomeric receptors participate in the processes. However, there is increasing evidence that GPCRs exist as dimers. For instance, we have shown that D1 and D2 dopamine receptors expressed in cell lines exist as homodimers (Ng et al., 1994; George et al., 1998) and that D2 dopamine receptors (D2DR) exist as dimers in human and rat brain tissue (Zawarynski et al., 1998). The M2 muscarinic (Maggio et al., 1993a), β2-adrenergic (Hebert et al., 1996), V2 vasopressin (Hebert et al., 1996), metabotropic glutamate (Romano et al., 1996), H2 histamine (Fukushima et al., 1997), δ-opioid (Cvejic and Devi, 1997), D3 dopamine (Nimchinsky et al., 1997), Ca²⁺-sensing (Bai et al., 1998), and B2 bradykinin (AbdAlla et al., 1999) receptors have also been shown to form dimers, suggesting that dimerization may be a universal aspect of GPCR biology. Furthermore, we have shown recently that two closely related serotonin receptor subtypes, the 5-HT₁B and 5-HT₁D receptors, form homodimers and undergo heterodimeric assembly when coexpressed (Xie et al., 1999). Hetero-oligomerization (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999) and homo-oligomerization (Ng et al., 1999) have also been shown to occur between the metabotropic γ-aminobutyric acid receptors GABA_BR1 and GABA_BR2.

One of the first observations suggesting that a monomeric model may not fully describe the mechanisms of GPCR function came from studies on muscarinic/adrenergic receptor chimera (Maggio et al., 1993a). Two chimera, both incapable of binding muscarinic or adrenergic ligands when expressed alone, recovered ligand binding properties when coexpressed. It was postulated that the two chimera associated and thereby reconstituted a ligand binding pocket. A similar

ABBREVIATIONS: GPCR, G protein-coupled receptor; D2DR, D2 dopamine receptor; TM, transmembrane; D2N, N terminus-truncated D2DR fragment (amino acids 1–373); D2C, C terminus-truncated D2DR fragment (amino acids 212–414); PCR, polymerase chain reaction; CHO, Chinese hamster ovary; PNR, putative neurotransmitter receptor; azido-YM, 4-azido-5-iodo-nemonapride; azido-NAPS, azidophenethyl-spiperone; CCR5, human chemokine receptor 5.

study using mutated type 1 angiotensin II receptors also showed recovery of ligand binding, albeit low, on coexpression of receptors incapable of binding when expressed alone (Monnot et al., 1996). Based on these studies and protein structure analysis, a model was proposed in which transmembrane (TM) helices of GPCRs participate in domain swapping when in the dimeric state, to explain how binding pockets are recovered in binding-incapable GPCRs (Gouldson et al., 1997). According to this model, on receptor dimerization, the original binding pockets of the two subunit monomers are replaced by the formation of two binding pockets that are similar in structure to the monomeric receptor, except that they are formed from regions donated by both monomers (see Fig. 1).

In this study, we examined TM domain swapping in D2DR dimerization, using D2DR point mutants and D2DR truncation mutants containing TM domains 1 to 5 or TM domains 6 and 7 (Fig. 2). We demonstrated that the mechanism of D2DR dimerization does not involve TM domain swapping as predicted by the above model. However, our observations using the mutant receptors confirmed that there was intermolecular association among D2DRs and showed that, because of this interaction, mutant D2DRs could antagonize wild-type D2DRs when coexpressed. Moreover, our data indicated that D2DR exists as oligomeric structures in the cell.

Materials and Methods

Construction of D2DR Point Mutants. The Asp residue in TM domain 3 (position 114) of the D2DR was mutated to Asn and is designated Asp[114]Asn. In the second mutant, two Ser residues in TM domain 5 (positions 194 and 197) were mutated to Ala residues and are designated Ser[194]Ala/Ser[197]Ala D2DR. The construction of the Asp[114]Asn D2DR and Ser[194]Ala/Ser[197]Ala D2DR mutant receptors

and their insertion into mammalian expression vectors have been described previously (Mansour et al., 1992).

Construction of D2DR and D2DR Fragment Expression Vectors. cDNA encoding the short isoform of the D2DR (kindly provided by Dr. O. Civelli, University of California Irvine, Irvine, CA) was used as the template in the polymerase chain reaction (PCR) construction of the cDNA constructs for the D2DR and the truncation mutants. The sequences (5′ to 3′) and designations of the oligonucleotides used were as follows: P191, GCAAGCTTGCC-ACCCAGTCGGTCCACCGC; P192, GCGCGGCCGCTCACTGAGTG-GCTTTCTTCTCCTTCTG; P780, GCAAGCTTGCCGCCATGGAA-AAGCGAGTCAACACCAAACGCAGCAGCCGAGC; P613, ACGCG-GCCGCAGGCTGCTGTGCGGGCAGGCACGAGAGTCAGCAGTGG-AGGATCTT.

The D2DR cDNA construct was generated using P191 and P613. The D2N cDNA that encoded amino acids 1 through 373 was generated using P191 and P192, and the D2C cDNA encoding amino acids 212 through 414 was generated using P780 and P613 (Fig. 2). PCR reactions utilized Pfu enzyme (Pharmacia, Piscataway, NJ) under the following conditions: 4-min precycle denaturation at 96°C followed by denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2.5 min for 30 cycles. All three PCR products were inserted into the mammalian expression vector pcDNA3. The absence of sequence errors and correct orientation of the PCR products into the expression vector were verified by sequencing.

Cell Culture and Transfection of Cells. COS-7 monkey kidney cells and Chinese hamster ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were maintained as monolayer cultures at 37°C in minimum essential medium supplemented with 10% fetal bovine serum and antibiotics. COS-7 or CHO-K1 cells were transiently transfected by the calcium phosphate precipitation method or by the use of lipofectamine reagent (Life Technologies, Rockville, MD). When a receptor, mutant receptor, or receptor fragment was expressed alone, an equal amount of pcDNA3 vector was cotransfected with each construct, so that the total amount of DNA
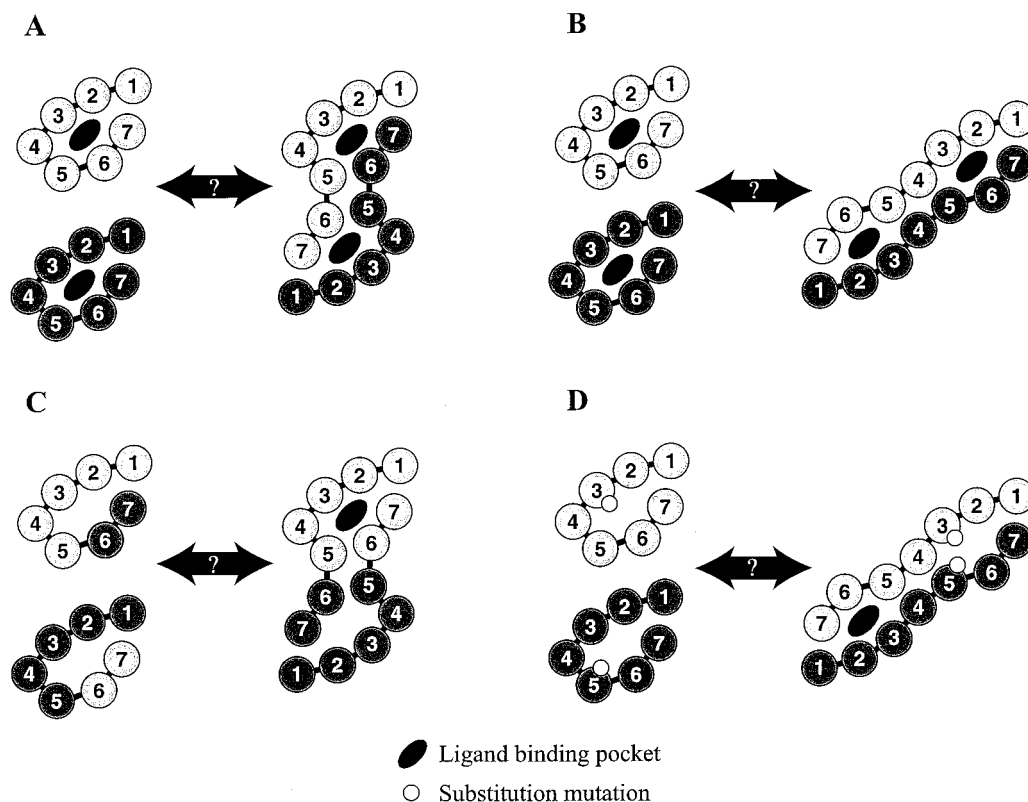
A   **B**

C   **D**

Fig. 1. Schematic representation of TM domain swapping model predicted for GPCRs (adapted from Gouldson et al., 1997). The illustration depicts a view of the receptors in the cell membrane from the extracellular domain. A, domain swapping of TM 1 to 5 from one receptor with TM 6 and 7 of another receptor. B, domain swapping of TM 1 to 4 from one receptor with TM 5 to 7 of another receptor. C, domain swapping between two receptor chimeras. Each chimera is incapable of forming a binding pocket alone, but an intact binding pocket is formed on dimerization in the manner shown in A. D, domain swapping between two receptor substitution mutants. Each mutant is incapable of binding as a result of the mutation, but an intact binding pocket is formed on dimerization in the manner shown in B.

● Ligand binding pocket

○ Substitution mutation

used was consistent with studies involving transfections with two constructs. When two constructs were used for coexpression experiments, equal amounts of each construct were used unless otherwise stated. For coexpression experiments in which the amount of one construct used was variable, the total amount of DNA transfected was kept constant by the addition of a compensating amount of pcDNA3 vector. The full-length D2DR was also coexpressed with the μ-opioid receptor or an orphan aminergic GPCR, putative neurotransmitter receptor (PNR), the cloning of which has been reported previously (Zeng et al., 1998).

Membrane Preparation. All tissues were washed extensively with PBS. Cell lysate was prepared by polytron disruption in ice-cold 5 mM Tris-HCl, 2 mM EDTA buffer, containing 5 μg/ml leupeptin, 10 μg/ml benzamidine, and 5 μg/ml soybean trypsin inhibitor as described previously (Ng et al., 1994). Membrane protein was deter-

Fig. 2. Schematic representation of the D2 dopamine receptor and the truncation mutants D2N and D2C.

Fig. 3. Receptor density determined by radioligand binding assay. [³H]Nemonapride saturation binding was performed on membranes from COS-7 cells transiently expressing D2DR, Asp[114]Asn D2DR, or Ser[194]Ala/Ser[197]Ala D2DR or coexpressing Asp[114]Asn D2DR and Ser[194]Ala/Ser[197]Ala D2DR.

mined by the Bradford assay according to the manufacturer's instructions (BioRad, Hercules, CA).

Receptor Pharmacology. Saturation binding experiments were performed with ~20 μg of membrane protein with increasing concentrations of [³H]spiperone or [³H]nemonapride (also known as YM-09151-2) (10–4000 pM, final concentration) and used to determine receptor densities (B_{max}) and affinities for ligands (K_D) as previously described (Ng et al., 1996). Competition experiments were done in triplicate in the absence or presence of 5′-guanylimidodiphosphate (100 μM, final concentration) with increasing concentrations of dopamine (10^{-12} to 10^{-3} M). The concentration of radioligand used in the competition assays was approximately equivalent to its K_D. Bound ligand was isolated by rapid filtration through a Brandel 48-well cell harvester, using Whatman GF/C filters. Data were analyzed by nonlinear least-squares regression, using the computer-fitting program Prism version 2.01 (GraphPad).

Photoaffinity Labeling. The precursor compounds for the photoaffinity label [125I]4-azido-5-iodo-nemonapride ([125I]azido-YM) and 125I-azidophenethyl-spiperone (125I-azido-NAPS) were supplied by Research Biochemicals Inc. (Natick, MA) as part of the Chemical Synthesis Program of the National Institute of Mental Health. Radioiodination was performed by NEN Life Science Products (Boston, MA). Photoaffinity labeling was performed as described previously (Ng et al., 1996). Briefly, membranes prepared from D2DR-expressing cells were incubated with [125I]azido-YM or 125I-azido-NAPS for 1.5 h. Specific binding was defined by coincubation with 1 μM (+)-butaclamol. The mixture was exposed to UV light to cross-link the photolabel compound, and the membrane was collected and subjected to electrophoresis. The gel was then fixed and exposed to film.

Adenylyl Cyclase Activity. Adenylyl cyclase assays were conducted essentially as described previously (Salomon et al., 1974). The assay mix contained 0.02 ml of membrane suspension (10–25 μg of protein), 0.012 mM ATP, 0.1 mM cAMP, 0.053 mM GTP, 2.7 mM phosphoenolpyruvate, 0.2 units of pyruvate kinase, 1 unit of myokinase, 1 μM forskolin, and 0.13 μCi of [32P]ATP in a final volume of 0.05 ml. The mixture was incubated with 10^{-12} to 10^{-3} M dopamine at 27°C for 20 min, and enzyme activities were determined. Data were analyzed by computer-fitted nonlinear least-squares regression.

Electrophoresis and Immunoblot Analysis. In brief, tissues were solubilized in sample buffer consisting of 50 mM Tris-HCl (pH 6.5), 1% SDS, 10% glycerol, 0.003% bromophenol blue, and 10% 2-mercaptoethanol. The samples were subjected to polyacrylamide gel electrophoresis with 12% acrylamide gels and electroblotted onto nitrocellulose as previously described (Ng et al., 1996). Unless otherwise indicated, D2DR immunoreactivity was revealed with the rabbit polyclonal antibody, which was raised against a 120-amino acid sequence (aa 221–340) in the third intracellular loop of the human D2DR (Levey et al., 1993). The antibody, designated AL-26,
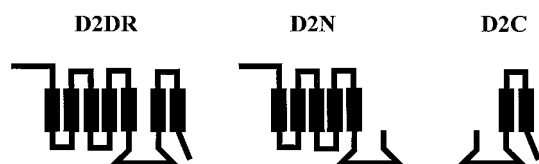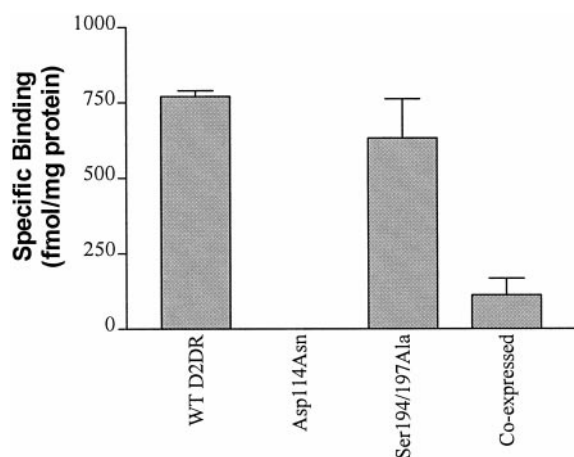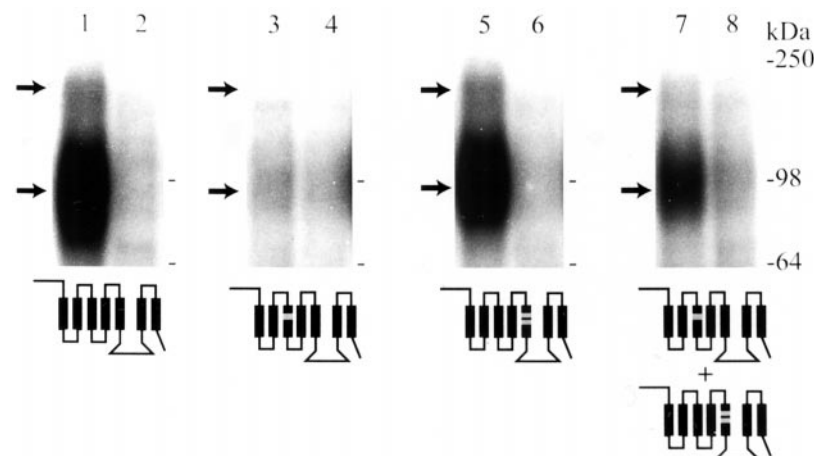

Fig. 4. Autoradiogram showing photoaffinity labeling by [125I]-azido-YM of membranes from COS-7 cells expressing D2DR (lanes 1 and 2), D2DR Asp[114]Asn D2DR (lanes 3 and 4), and D2DR Ser[194]Ala/Ser[197]Ala D2DR (lanes 5 and 6), and coexpressing D2DR Asp[114]Asn D2DR and D2DR Ser[194]Ala/Ser[197]Ala D2DR (lanes 7 and 8). Lanes 1, 3, 5, and 7 show total labeling. Lanes 2, 4, 6, and 8 show nonspecific labeling, as defined by 1 μM (+)-butaclamol. Arrows indicate the photolabeled receptor in lanes 1, 3, 5, and 7. For each labeling condition, 50 μg of protein was used. The autoradiogram shown is from a 3-day exposure and is representative of two independent experiments.

was a generous gift from Dr. Mark R. Brann (Acadia Pharmaceuticals Inc., San Diego, CA). An antibody recognizing the N terminus of the human D2DR, N-19 (Santa Cruz Biotechnology, Santa Cruz, CA), was also used.

Densitometry. The relative intensities of bands visualized by immunostaining and in autoradiograms were determined using reflective densitometry and the Gel Doc 1000 Video Documentation System and Molecular Analyst software (BioRad).
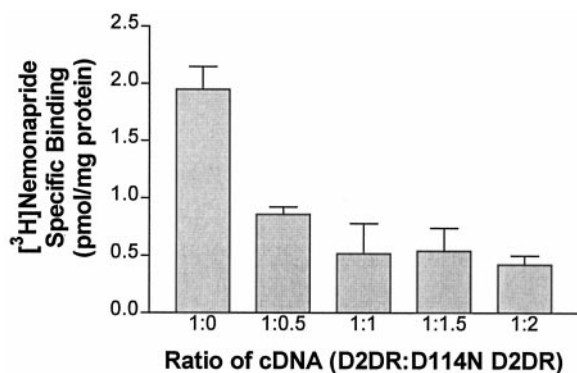
Results

Coexpression of Asp[114]Asn and Ser[194]Ala/Ser[197]Ala D2DR Mutants. Radioligand binding with the agonists [3H]dopamine and [3H]N-propyl-apomorphine and with the antagonist [3H]nemonapride was undetectable in cells expressing Asp[114]Asn D2DR (Fig. 3). In cells expressing the Ser[194]Ala/Ser[197]Ala D2DR, agonist binding was diminished compared with wild-type D2DR, but nemonapride binding was not significantly different (Fig. 3). These binding characteristics are consistent with previous studies on these D2DR mutants (Mansour et al., 1992). We hypothesized that, as a result of TM domain swapping, the number of detected binding sites would increase on coexpression of Asp[114]Asn D2DR with Ser[194]Ala/Ser[197]Ala D2DR because of the reconstitution of intact binding pockets (Fig. 1D). However, when the point mutants were coexpressed, radioligand-detected receptor density was significantly diminished compared with that of cells only expressing Ser[194]Ala/Ser[197]Ala D2DR. Photoaffinity labeling with [125I]azido-YM (Fig. 4) was consistent

Fig. 5. D2DR density detected by [3H]nemonapride binding in membranes from COS-7 cells transiently transfected with D2DR cDNA and increasing amounts of Asp[114]Asn D2DR cDNA. The ratio of D2DR cDNA to Asp[114]Asn D2DR cDNA is indicated. For all conditions, the amount of D2DR cDNA used for transfection was constant. The total amount of DNA used for each transfection condition was also kept constant by the addition of an appropriate amount of pcDNA3 vector.
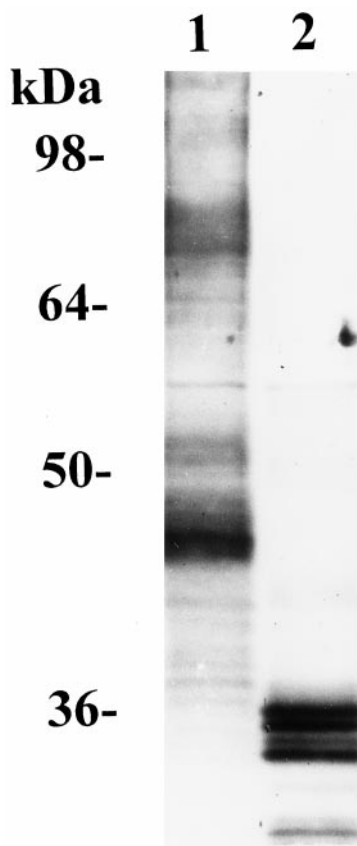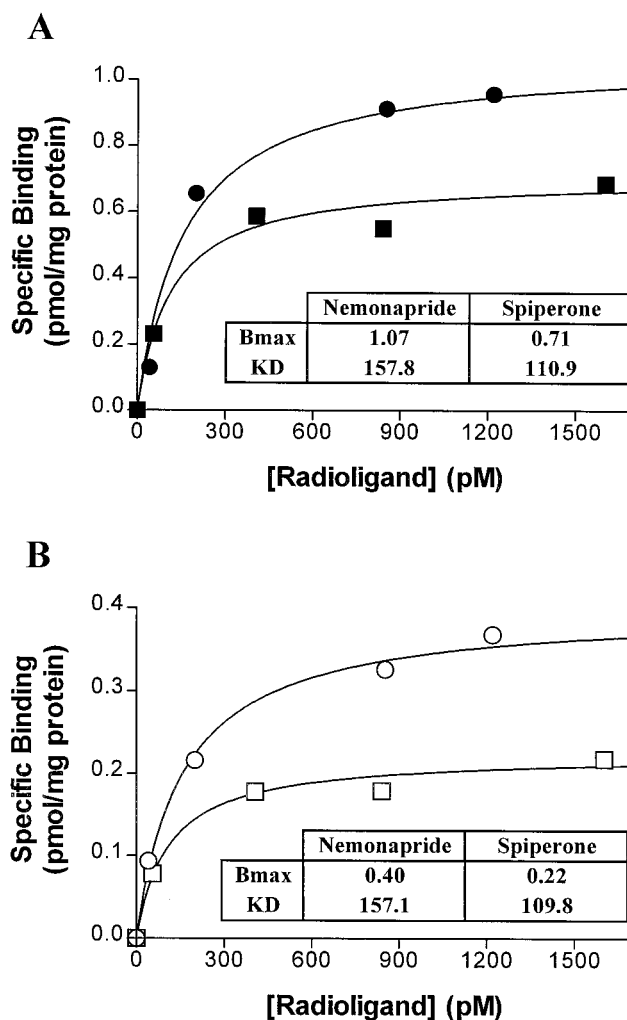
Fig. 7. Saturation isotherms of [3H]spiperone- (■, □) and [3H]nemonapride- (●, ○) specific binding in membranes prepared from COS-7 cells transiently transfected with D2DR cDNA (A) and from COS-7 cells transiently transfected with D2N and D2C cDNA (B). The results shown are from one of five independent experiments. The receptor density estimates (B_{max}) (in pmol/mg protein) and dissociation constants (K_D) (in pM) calculated from the binding data are shown in the inset tables.

Fig. 6. Immunoblot analysis of membranes from COS-7 cells transiently transfected with cDNA encoding D2N (lane 1) and D2C (lane 2). For each lane, 25 μg of protein was used. The immunoblot shown is representative of two independent experiments.

with the radioligand binding data. Densitometry showed that labeling of receptor monomer was reduced by ~60% in cells coexpressing Asp[114]Asn D2DR and Ser[194]Ala/Ser[197]Ala D2DR compared with cells expressing Ser[194]Ala/Ser[197]Ala D2DR alone and that there was a ~70% reduction in labeling of the receptor dimer. Because the number of ligand binding sites was not increased or even maintained, it was concluded that that the model of TM domains 1 to 4 swapping with TM domains 5 to 7, although adequate to explain the results obtained with the angiotensin II receptor (Monnot et al., 1996; Gouldson et al., 1997), does not accurately describe dimerization in the D2DR.

Coexpression of Asp[114]Asn D2DR and Wild-Type D2DR. Because coexpression of the Asp[114]Asn D2DR with the Ser[194]Ala/Ser[197]/Ala D2DR decreased the radioligand-detected receptor density, we examined the effects of Asp[114]Asn D2DR on the wild-type receptor. When Asp[114]Asn D2DR was coexpressed with wild-type D2DR, radioligand-detected receptor density was attenuated (Fig. 5). As the ratio of Asp[114]Asn D2DR cDNA to wild-type D2DR cDNA used for transfection increased, the degree of binding inhibition also increased.

N-Terminal and C-Terminal D2DR Fragments Are Properly Processed and Trafficked. To determine whether TM domains 1 to 5 participated in domain swapping with TM domains 6 to 7, we used a strategy involving receptor fragments. However, it was necessary to demonstrate that receptor fragments were properly folded and capable of mimicking the appropriate portions of the wild-type receptor. D2N, which includes TM domains 1 to 5, or D2C, which includes TM domains 6 and 7 (see Fig. 2), were transiently expressed separately in COS-7 cells. Immunoblot analyses of membranes from these cells (Fig. 6) revealed that D2N and D2C were highly expressed and trafficked to the cell surface. In membranes from cells expressing D2N, bands of ~40 kDa, ~55 kDa, and ~75 kDa were detected (Fig. 6, lane 1). The immunoreactive species of ~75 kDa represented the glycosylated form of this truncated receptor, and the ~40 kDa and ~55 kDa bands likely represented nonglycosylated or partially glycosylated receptor. The addition of ~35 kDa is consistent with the molecular weight change in the full-length D2DR due to glycosylation (Grigoriadis et al., 1988). An immunoreactive band of ~35 kDa representing D2C was observed in membranes from cells expressing D2C (Fig. 6, lane 2). No higher molecular weight species were present, because D2C does not have any N-linked glycosylation sites.

There was no detectable binding in cells expressing either D2N or D2C (data not shown). However, the coexpression of D2N and D2C resulted in the appearance of saturable and specific binding of the D2 antagonists [3H]spiperone and [3H]nemonapride with affinity constants similar to those derived from binding to full-length D2DR (Fig. 7). When binding assays were performed on membranes from cells expressing D2N mixed with membranes from cells expressing D2C, no specific binding was detected (data not shown).

The labeling of membranes expressing D2N and D2C singly or together by [125I]azido-YM is shown in Fig. 8A. The photoaffinity ligand did not specifically label either of the receptor fragments when expressed separately (Fig. 8A, lanes 1 and 3). However, when D2N and D2C were coexpressed, D2N was specifically labeled (Fig. 8A, lane 5). Monomeric D2DR and dimeric D2DR (Fig. 8B, lane 1) were specifically labeled by [125I]azido-YM in D2DR-expressing cell membranes (Fig. 8A, lane 7). These data indicate that when D2N and D2C were coexpressed, the D2N fragments assembled with D2C fragments to form functional binding pockets but dissociated during SDS polyacrylamide gel electrophoresis.

Agonist competition of radiolabeled antagonist binding indicated that ~30% of the full-length D2DRs expressed in COS-7 cells were in a high affinity state (Fig. 9A). Dopamine competition of [3H]spiperone binding to membranes prepared from COS-7 cells coexpressing D2N and D2C was also best fitted to a model with two affinity states (Fig. 9B). The full-length receptor and coexpressed D2N and D2C exhibited agonist-detected high-affinity binding that was guanine nucleotide-sensitive (data not shown), suggesting association of the reconstituted receptor with G protein. To confirm the association of the fragments into fully functional receptors, D2N and D2C were coexpressed in CHO-K1 cells for adenylyl cyclase studies. The reconstituted receptor fragments were able to mediate dopamine-induced inhibition of adenylyl cyclase (Fig. 9C). These results are not unexpected, as split receptor association has been shown for fragments of other GPCRs (Kobilka et al., 1988; Maggio et al., 1993b; Ridge et al., 1995; Schoneberg et al., 1996; Saunders et al., 1998) and for the related bacteriorhodopsin (Marti, 1998). However, these data are significant because, taken together, the immunoblot analyses, the binding data, and the adenylyl cyclase assay indicate that D2N and D2C properly mimic portions of the full-length D2DR.

Coexpression of D2N and Asp[114]Asn D2DR. We predicted that if domain swapping of TM domains 1 to 5 with TM domains 6 and 7 occurred as postulated for the muscarinic/
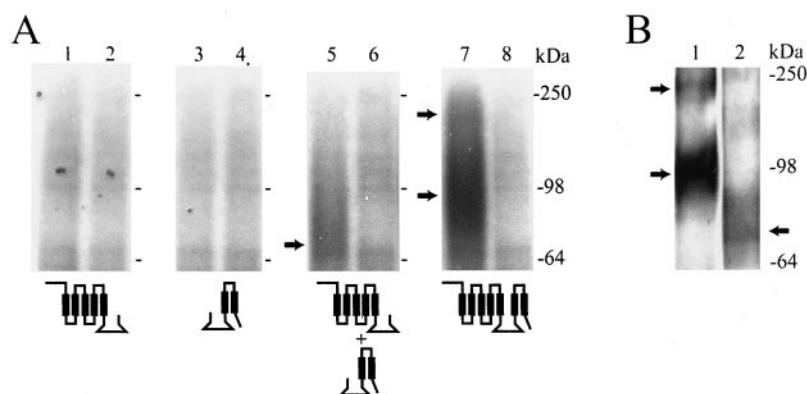

Fig. 8. A, autoradiogram showing photoaffinity labeling by [125I]azido-YM of membranes from COS-7 cells expressing D2N (lanes 1 and 2), D2C (lanes 3 and 4), and D2DR (lanes 7 and 8), and coexpressing D2N and D2C (lanes 5 and 6). Lanes 1, 3, 5, and 7 show total labeling. Lanes 2, 4, 6, and 8 show nonspecific labeling, as defined by 1 μM (+)-butaclamol. Arrows indicate the D2DR monomer and dimer in lane 7 and D2N in lane 5. For each labeling condition, 50 μg of protein was used. The autoradiogram shown is from a 3-day exposure and is representative of three independent experiments. B, Immunoblot analysis of membranes from COS-7 cells transiently expressing D2DR (lane 1) or D2N (lane 2). Arrows indicate D2DR monomer and dimer (lane 1) and D2N monomer (lane 2). For each lane, 25 μg of protein was used. The immunoblot shown is representative of three independent experiments.
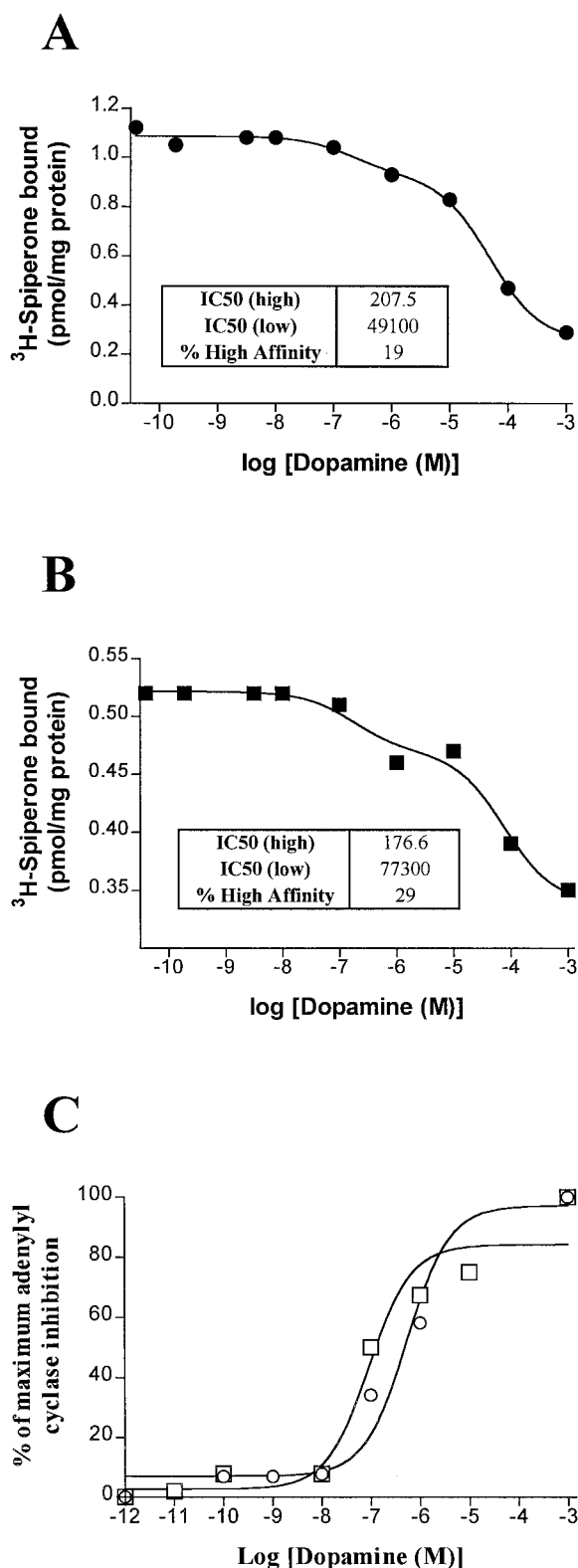
A

B

C

Fig. 9. Competition of [^{3}H]spiperone binding with dopamine in membranes prepared from COS-7 cells transiently transfected with D2DR cDNA (A) and from COS-7 cells transiently transfected with D2N and D2C cDNA (B). The results shown are from one of four independent experiments. The IC$_{50}$ value (in nM) for the high- and low-affinity binding states and the percentage of high-affinity states calculated from the binding data are shown in the inset tables. C, dopamine-mediated adenylyl cyclase inhibition in forskolin-stimulated membranes from CHO cells expressing the D2DR (○) or coexpressing D2N and D2C (□). The maxi-

adrenergic receptor chimera (Maggio et al., 1993a; Gouldson et al., 1997), coexpression of D2N with Asp[114]Asn would result in the recovery of D2DR binding (Fig. 10). Functional rescue of a defective mutant V2 vasopressin receptor has been shown by coexpression of a truncation mutant (Schoneberg et al., 1996). To determine whether the coexpression of Asp[114]Asn D2DR with D2N could result in the recovery of ligand binding, cells coexpressing the receptor fragment and the point mutant were subjected to saturation binding assays using [^{3}H]N-propyl-apomorphine and [^{3}H]nemonapride. No specific binding was detected (data not shown), indicating that TM domains 1 to 5 do not participate in swapping with TM domains 6 and 7 in the D2DR.

Coexpression of Full-Length D2DR with the Truncated Receptors Inhibited Receptor Function. Coexpression of the full-length D2DR with either the D2N or D2C in COS-7 cells resulted in a significant reduction in [^{3}H]spiperone and [^{3}H]nemonapride binding (Table 1). This antagonism increased as the ratio of receptor fragment to D2DR increased (Fig. 11). A reduction in photoaffinity ligand binding in the presence of receptor fragment was also observed. An almost complete attenuation of azido-NAPS binding was observed in photolabeled membranes coexpressing the D2DR with either D2N or D2C (Fig. 12A, lanes 5 and 7). Radioli-
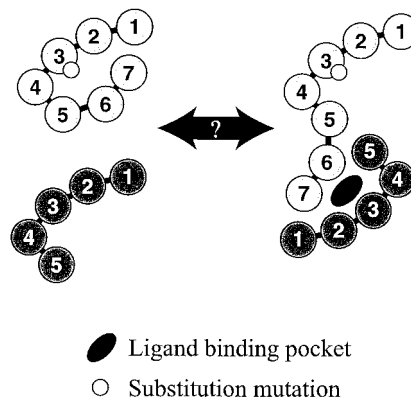

⬛ Ligand binding pocket

○ Substitution mutation

Fig. 10. Schematic representation of TM domain swapping between a GPCR with a mutation in TM 3 with a GPCR fragment containing TM domains 1 to 5 (modified from Gouldson et al., 1997). The receptor mutants are incapable of binding, but an intact binding pocket is formed on dimerization.

TABLE 1
Receptor density detected by radioligand binding assay.

[^{3}H]Spiperone and [^{3}H]nemonapride saturation binding was performed on membranes from COS-7 cells transiently expressing D2DR, coexpressing D2DR and D2N, or coexpressing D2DR and D2C. For the cells expressing D2DR only, an equal amount of expression vector containing no insert was cotransfected with the D2DR cDNA. For cells coexpressing D2DR and truncated receptor, the ratio of wild-type receptor cDNA to mutant receptor cDNA was 1:1.

Protein expressed in COS-7 cell	B_{max}	
	Spiperone	Nemonapride
	pmol/mg protein	
D2DR	0.42 ± 0.03	0.63 ± 0.03
D2DR + D2N	0.04 ± 0.01	0.06 ± 0.01
D2DR + D2C	0.14 ± 0.06	0.30 ± 0.08

mum inhibition of adenylyl cyclase activity, as a percentage of forskolin-stimulated activity, was 22.2% ± 1.6 for D2DR ($n = 3$) and 9.84% ± 3.14 ($n = 3$) for coexpressed D2N and D2C. The results shown are from one of three independent experiments.

gand binding to the D2DR was not altered when membranes from cells expressing only D2DR were mixed with membranes from cells expressing only D2N or D2C (data not shown).
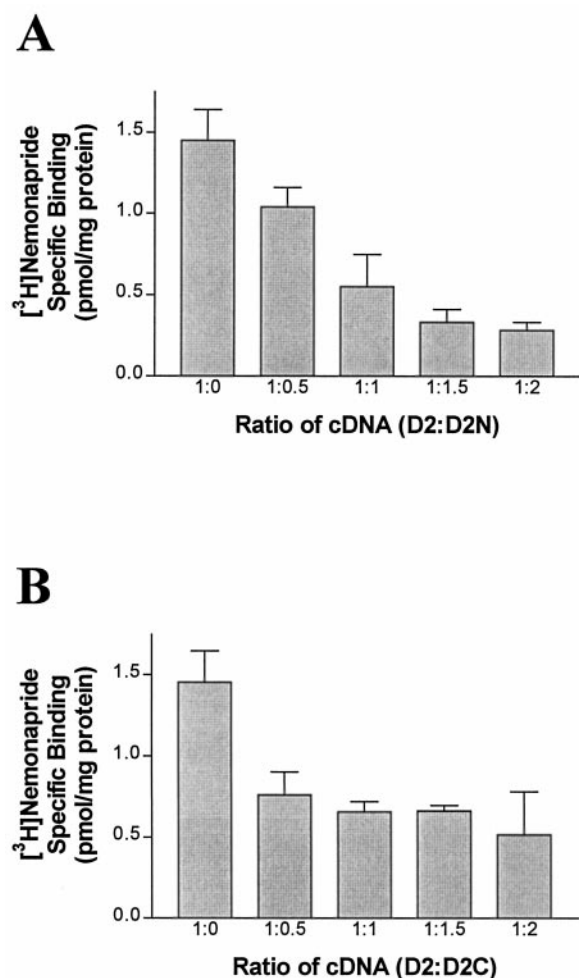

Fig. 11. D2DR density detected by [³H]nemonapride binding in membranes from COS-7 cells transiently transfected with D2DR cDNA and increasing amounts of D2N cDNA (A) and D2DR cDNA and increasing amounts of D2C cDNA (B). The ratio of D2DR cDNA to D2N or D2C cDNA is indicated. For all conditions, the amount of D2DR cDNA used for transfection was constant. The total amount of DNA used for each transfection condition was also kept constant by the addition of an appropriate amount of pcDNA3 vector.

Radioligand binding was attenuated by ~90% when the D2DR was coexpressed with D2N and by ~55 to 65% when it was coexpressed with D2C. Densitometry of photoaffinity labeling of the D2DR monomer showed a ~95% reduction in labeling by D2N and a ~85% reduction by D2C. Furthermore, the coexpression of the full-length D2DR together with either D2N or D2C in CHO-K1 cells resulted in the loss of adenylyl cyclase inhibition by dopamine (data not shown).

Immunoblot analyses of membranes from cells coexpressing full-length D2DR and D2C using an antibody directed against the N terminus of the D2DR demonstrated that cell surface expression of the D2DR was reduced as D2C expression increased (Fig. 13A). Immunoblots of the same membranes, using AL-26 antibody, revealed that cell surface expression of D2C increased as the ratio of D2C cDNA to D2DR cDNA used in the transfections increased (Fig. 13B). This suggested that trafficking of the D2DR was increasingly impaired as the expression of D2C increased but that protein expression and trafficking mechanisms in the cell were generally unaffected.

Coexpression of the D2DR with Another GPCR. To determine whether the antagonism by the receptor fragments or receptor mutant was due to the cotransfection with the cDNA of a related membrane protein, cells were cotransfected with D2DR cDNA and the cDNA for μ-opioid receptor or an orphan GPCR, PNR (Zeng et al., 1998). Radioligand binding, photoaffinity labeling, and adenylyl cyclase inhibition by dopamine were not significantly different in membranes from cells transfected with D2DR cDNA and plasmid DNA compared with membranes from cells cotransfected with D2DR cDNA and μ-opioid receptor cDNA or PNR cDNA (data not shown).

Discussion

In this study we have shown that coexpression of a mutant D2DR incapable of receptor function with the wild-type D2DR results in attenuation of binding and function of the wild-type receptor. It was observed that coexpression of the Asp^{114}Asn D2DR with the Ser^{194}Ala/Ser^{197}Ala D2DR decreased the radioligand-detected receptor density. We therefore postulated that nonfunctional receptors may act as antagonists of wild-type receptors. To further test this, the Asp^{114}Asn D2DR, D2N, and D2C were each coexpressed with wild-type D2DR. In each case, receptor function was attenu-
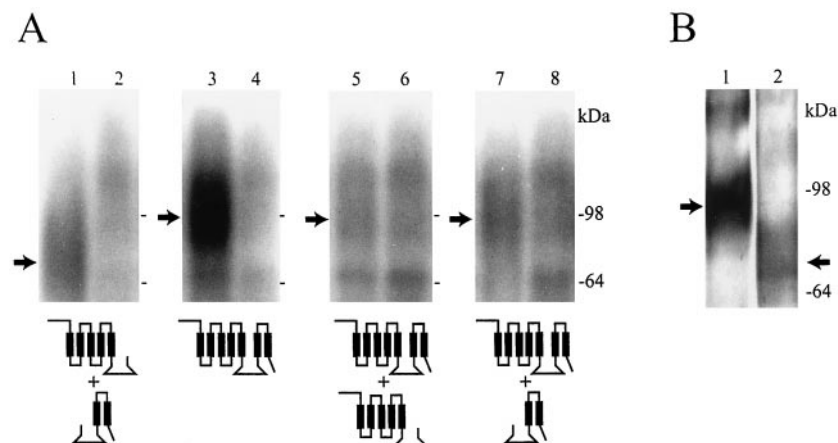
Fig. 12. A, autoradiogram showing photoaffinity labeling by ^{125}I-4-azido-NAPS of membranes from COS-7 cells coexpressing D2N and D2C (lanes 1 and 2), D2DR and D2N (lanes 5 and 6), and D2DR and D2C (lanes 7 and 8), and expressing D2DR (lanes 3 and 4). Lanes 1, 3, 5, and 7 show total labeling. Lanes 2, 4, 6, and 8 show nonspecific labeling, as defined by 1 μM (+)-butaclamol. Arrows indicate the D2DR monomer in lanes 3, 5, and 7 and D2N in lane 1. For each labeling condition, 50 μg of protein was used. The autoradiogram shown is from a 4-day exposure and is representative of three independent experiments. B, immunoblot analysis of membranes from COS-7 cells transiently expressing D2DR (lane 1) or D2N (lane 2). Arrows indicate D2DR monomer (lane 1) and D2N monomer (lane 2). For each lane, 25 μg of protein was used. The immunoblot shown is representative of three independent experiments.

ated, compared with the wild-type receptor expressed alone, in an expression-dependent manner. There are numerous reports of mutant and truncated GPCRs that have diminished or no function, both as naturally occurring phenomena and as the result of gene manipulation. However, there are few reports concerning the coexpression of these mutant receptors with the wild-type receptor. It has been demonstrated that a truncation mutant of the human chemokine receptor 5 (CCR5) can inhibit CCR5-mediated human immunodeficiency virus infection in individuals who are heterozygous for the mutant GPCR (Benkirane et al., 1997). Recently, antagonism of the V2 vasopressin receptor by receptor fragments has been shown (Zhu and Wess, 1998).

In our studies, photoaffinity labeling of membranes where the D2DR was coexpressed with D2N or D2C revealed that the number of monomeric binding sites was markedly reduced (Fig. 8A). It was shown that this loss of binding sites was not the result of indirect effects on protein processing pathways (Fig. 13). This suggested that direct interactions between the D2DR and the mutant receptor resulted in the loss of receptor function and/or a reduced efficiency in trafficking of the wild-type receptor to the cell surface. One would expect that if a proportion of the D2DR functioned in the cell in a monomeric state, trafficking and function of the D2DR monomers would be unaffected by the presence of the receptor fragment. However, because cell surface expression of the receptor monomer was decreased, it can be concluded that the D2DRs do not function as monomers. Therefore, it can be inferred that D2DRs exist only as oligomers in the cell and that the detection of a receptor species corresponding in molecular weight to a receptor monomer may be the result of dissociation of the oligomer during electrophoresis.

A similar conclusion that D2DRs exist as oligomers can be drawn from the point mutant D2DR coexpression. The mutant Asp^{114}Asn D2DR is incapable of binding ligand because of the substitution of the critical amino acid in TM domain 3. When this mutant was coexpressed with Ser^{194}Ala/Ser^{197}Ala

D2DR, which, when expressed alone, bound antagonist with high affinity, the number of binding sites was decreased. In this case also, photoaffinity labeling confirmed attenuated binding to both dimeric and monomeric binding sites. This indicated that the function of the visualized monomeric and dimeric Ser^{194}Ala/Ser^{197}Ala D2DR was impaired. Therefore, one must conclude that the monomers, as well as the receptor dimers, were associated with the nonbinding mutant receptor in the cell, suggesting that the association of a binding-incapable receptor mutant with a binding-intact receptor mutant caused the loss of function. Thus our data suggest that an oligomeric array of intact receptors is necessary for normal function.

The possibility that the D2DR exists only as oligomeric complexes in the cell is not unexpected. It has been predicted, based on radioligand binding data, that there is cooperation between the binding sites in the muscarinic receptor, which can be explained if the receptors exist as oligomers (Wreggett and Wells, 1995; Chidiac et al., 1997). It has been shown that multiple TM domain-spanning proteins such as the band 3 erythrocyte protein exist in large oligomeric arrays on the cell surface (Casey and Reithmeier, 1991). Furthermore, it is thought that G proteins may also exist in large oligomeric structures (Jahangeer and Rodbell, 1993). Oligomerization and the disruption of cell surface targeting may be the mechanism by which the mutant receptor antagonism occurs.

Our future studies will attempt to elucidate the precise sites of interaction involved in GPCR dimerization. It will be interesting to determine whether more than one mechanism is involved in the formation of receptor oligomers. Understanding dimerization may provide key insights into diseases involving both functional and defective GPCRs.

In summary, we have shown that TM domain swapping does not appear to occur in D2DR dimerization. Furthermore, we demonstrated that when a D2DR fragment was expressed with the full-length D2DR, or a mutant receptor incapable of ligand binding was coexpressed with the wild-type D2DR, receptor function was antagonized. We conclude that this disruption of the functional receptor is the result of an interaction between the receptor mutant and the D2DR that results from a disturbance in the ordering of the oligomeric receptor complex and a failure to efficiently express on the cell surface. A properly arranged oligomeric complex appeared to be required for D2DR trafficking. Therefore, we propose that active D2DR only exists as oligomers in the cell.

Fig. 13. Immunoblot analyses of membranes from COS-7 cells transiently transfected with cDNA encoding D2DR (lanes 1 and 6) or with D2DR cDNA and D2C cDNA in 1:0.5 (lane 2 and 7), 1:1 (lane 3 and 8), 1:1.5 (lane 4 and 9), and 1:2 (lane 5 and 10) ratios. A, immunodetection using N19 antibody directed against the N terminus of the D2DR. Arrows indicate monomeric and dimeric D2DR. B, immunodetection using AL-26 antibody. For each lane, 25 μg of protein was used.

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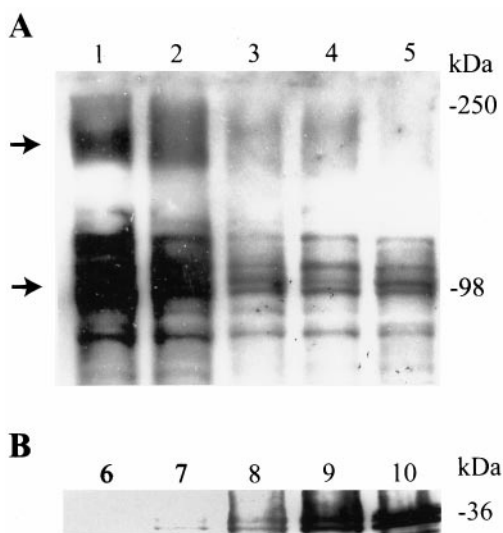
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