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Complementary roles of the DRY motif and C-terminus tail of GPCRS for G protein coupling and β-arrestin interaction

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Abstract

β-arrestin mediates the desensitization of GPCRs and acts as an adaptor molecule to recruit the receptor complex to clathrin-rich regions. Class-A GPCRs subsequently dissociate from β-arrestin but class-B GPCRs internalize with β-arrestin in the endocytic vesicles. Here the dopamine D_2 and D_3 receptors, which have similar structural features but different intracellular trafficking properties, were used in an attempt to better understand the structural requirements for the classification of GPCRs. The C-terminus tail of the vasopressin type-2 receptor was added to the ends of D_2R and D_3R to increase their affinity to β-arrestin. A point mutation was introduced into the DRY motif to change their basal activation levels. Among a battery of constructs in which the C-terminus tail and/or DRY motif was altered, class-B behavior was observed with the constructs whose affinities for β-arrestin were increased complementarily and their signaling was either maintained or regained. In conclusion, the DRY motif and C-terminal tail of the GPCRs determine complementarily their intracellular trafficking behavior by regulating the affinity to β-arrestin and G protein coupling.

Keywords: Dopamine D_2 and D_3 receptor; DRY motif; β-arrestin; G protein

β-arrestin is recruited to the intracellular loop or C-terminal residues of the GPCRs, which are phosphorylated by GPCR kinase (GRK) [1,2]. β-arrestin mediates the uncoupling of GPCRs from the G protein and links the receptor protein to the endocytotic machinery by acting as an adaptor molecule to recruit receptor proteins to the clathrin-rich regions of the plasma membrane [3]. Two distinct intracellular trafficking patterns of GPCRs have been reported. β-arrestin binds to the class-A GPCRs with low affinity and is subsequently released after targeting the receptor to the clathrin-coated pits. In contrast, class-B receptors such as the angiotensin II type-1A receptor ($AT1_AR$) and vasopressin type-2 receptor (V_2R), show much stronger and more prolonged binding to β-arrestin. The class-B GPCRs have a relatively long C-terminal tail with multiple putative phosphorylation sites for GRKs. After recruitment to the clathrin-coated pits, the class-B receptors and β-arrestin remain bound together on the surface of the endocytic vesicles [4]. However, it is unclear if the C-terminal tail of the class-B GPCRs is enough to convert all the class-A GPCR to class-B or other receptor regions are required.

The Asp-Arg-Tyr (DRY) motif is located at the beginning of the second intracellular loop. Arg plays important roles in G protein coupling, receptor phosphorylation, and β-arrestin translocation by forming intramolecular interactions [5–7]. Therefore, it is believed that the Arg residue somehow constrains the receptors in the active or inactive conformations, depending on the receptor types concerned [8]. Therefore, the DRY motif is believed to be a key region that controls the basal activation levels of GPCRs.

The dopamine D_2 and D_3 receptors (D_2R, D_3R) have similar structural features but different intracellular trafficking properties [9,10]. D_2R and D_3R have virtually identical signaling pathways except that the D_2R signals 2–5

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times more efficient than D_3R [11]. Since the C-terminal tails of D_2R and D_3R are similar but show 2–5 times different signaling efficiencies, it was hypothesized that their distinct intracellular trafficking properties could be mediated by the different roles of the DRY motif, which would determine the basal activation levels of the GPCRs. The aim of this study was to understand the more fundamental principles that determine the intracellular trafficking properties of GPCRs, i.e., either class-A or class-B. This study was performed through comparative characterization of the signaling and intracellular trafficking of D_2R and D_3R, where the Arg residue in the DRY motif is mutated to His and/or the C-terminus tail of the vasopressin type-2 receptor was added to their C-terminal ends. The results show that the DRY motif and C-terminus tail have complementary roles in determining G protein coupling and intracellular trafficking behaviors by providing high affinity for β-arrestin and by determining the activation levels of GPCRs, respectively. Overall, it is the conformation of the receptor proteins that is important for determining their signaling and intracellular trafficking properties rather than the primary sequence.

Materials and methods

Materials. Human embryonic kidney cells (HEK-293) were obtained from the American Type Culture Collection (Rockville, MD, USA). Dopamine (DA), haloperidol, and the anti-FLAG M2 antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]-sulpiride, [^{35}S]-GTPγS, and [^{32}P]-orthophosphate were purchased from NEN (Boston, MA, USA), and [^{3}H]-spiperone was purchased from Amersham (Piscataway, NJ, USA). The antibodies for β-arrestin were provided by Dr. Lefkowitz (Duke University Medical Center). Common antibodies, such as horse peroxidase conjugated anti-mouse IgG and anti-goat IgG were purchased from Amersham Biosciences (Piscataway, NJ, USA).

Plasmids. The mammalian expression constructs for the wild-type human dopamine D_2 receptor (short form; D_2R) and D_3 receptor (D_3R) subtypes, G protein-coupled receptor kinase 2 (GRK2), β-arrestins2, and β-arrestin2-GFP are described elsewhere [10,12,13]. The R → H mutants in the DRY motif of D_2R and D_3R were prepared by site-directed mutagenesis. The chimeric receptors with the C-terminal tail of the vasopressin type-2 receptor contained the full D_2R or D_3R with the last 29 amino acids of the C-terminal tail of the vasopressin type-2 receptor (V_2Rt) (Ala-343-Ser-371) [4]. The chimeric receptor of D_3R whose 2nd and 3rd intracellular loops are replaced with those of D_2R (D_3-(IL23-D_2), was described in our previous studies [10,14].

Whole-cell phosphorylation. One day after transfection, the HEK-293 cells were seeded at a density of $\sim 1 \times 10^6$ cells/25-mm well, and phosphorylation assays were performed, as previously described [10].

GTP-γS binding studies. HEK-293 cells were homogenized with 3-4 five-second bursts of a Polytron homogenizer (setting 12,000 rpm, with 5 s on ice between bursts) in 20 mM Hepes/Na-Hepes, pH 7.4, 10 mM EDTA plus the protease inhibitors. The homogenates were centrifuged at 16,000*g* for 15 min at 4 °C, and the pellet was washed with the same buffer, centrifuged, and resuspended in 20 mM Hepes/Na-Hepes, pH 7.4, 0.1 mM EDTA. GTP-γS binding was carried out as previously described [15].

Confocal microscopy. For the β-arrestin translocation assays, the HEK-293 cells were transfected with the β-arrestin 2-GFP and the corresponding receptor constructs. One day after transfection, the cells were seeded onto 35-mm dishes containing a centered, 1-cm glass coverslip and allowed to recover for one day. The cells were incubated with 2 ml MEM containing 20 mM Hepes, pH 7.4, and examined using a Zeiss laser scanning confocal microscope.

Immunoprecipitation. Cells expressing the Flag-tagged receptor proteins were lyzed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) on a rotation wheel for 1 h at 4 °C. The supernatants were mixed with 35 μl of a 50% slurry of Flag-agarose beads between 2–3 h on the rotation wheel. The beads were washed three times for 10 min each with a washing buffer (50 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% NP-40). The immunoprecipitates were analyzed on SDS–PAGE and immunoblotted.

Sequestration assay. Two different strategies were used, either by taking advantage of the hydrophilic properties of sulpiride or using flow cytometry, as described elsewhere [10,13].

Results

Differential effects of the DRY motif and C-terminal tail of V_2R on the intracellular trafficking properties of D_2R and D_3R

Some class-A GPCRs can be converted to class-B by attaching the C-terminus tail of the type-B GPCR to their C-terminal ends. For example, the C-terminus tail of the vasopressin type-2 receptor (V_2Rt) has three sets of serine clusters that are important for β-arrestin binding, and V_2Rt has been used to increase the affinity of β-arrestin for GPCRs [4,16]. However, it is unclear if just adding the C-terminus tail of class-B GPCR is enough to switch any class-A GPCR to a class-B GPCR, or other receptor regions of class-A GPCRs play important roles. This is an important issue in current GPCR research because it was reported that the receptor/β-arrestin scaffolded signaling complex needs to be internalized in order to mediate G protein-independent ERK activation [17].

As previously shown, the intracellular trafficking of D_2R and D_3R were different. The robust agonist-induced translocation of β-arrestin, which is a hallmark for the homologous desensitization of many GPCRs [18], was observed in cells expressing D_2R during a short-term (Fig. S1, 5 min) or long-term stimulation (Fig. 1A1, left panel, 1 h). In contrast, the D_3R-mediated translocation of β-arrestin was only transiently observed (Fig. S1, 5 min; Fig. 1A1, right panel, 1 h). Fig. 1A1 shows that D_2R and D_3R belong to class-A GPCR because β-arrestin failed to undergo endocytosis with receptor proteins. Please note that the internalization of D_2R (Fig. S2A), and to a less extent D_3R, becomes evident when the cells are stimulated for 1 h as in Fig. 1A [10].

The addition of V_2Rt to D_2R (D_2R–V_2Rt) failed to switch the intracellular trafficking properties of D_2R from class-A to class-B, i.e., most of the β-arrestins fell off from the endocytic complex at the plasma membrane before the receptor protein moved from the plasma membrane to the cytoplasm (compare Fig. 1A1 and A2, left panels). On the other hand, the addition of V_2Rt switched D_3R from class-A to class-B, i.e., D_3R–V_2Rt co-traveled with β-arrestin in the endocytic vesicles (compare Fig. 1A1 and A2, right panels). This suggests that factors other than the intensity of the agonist-induced translocation of β-arrestin play roles in the determination of GPCR classes.

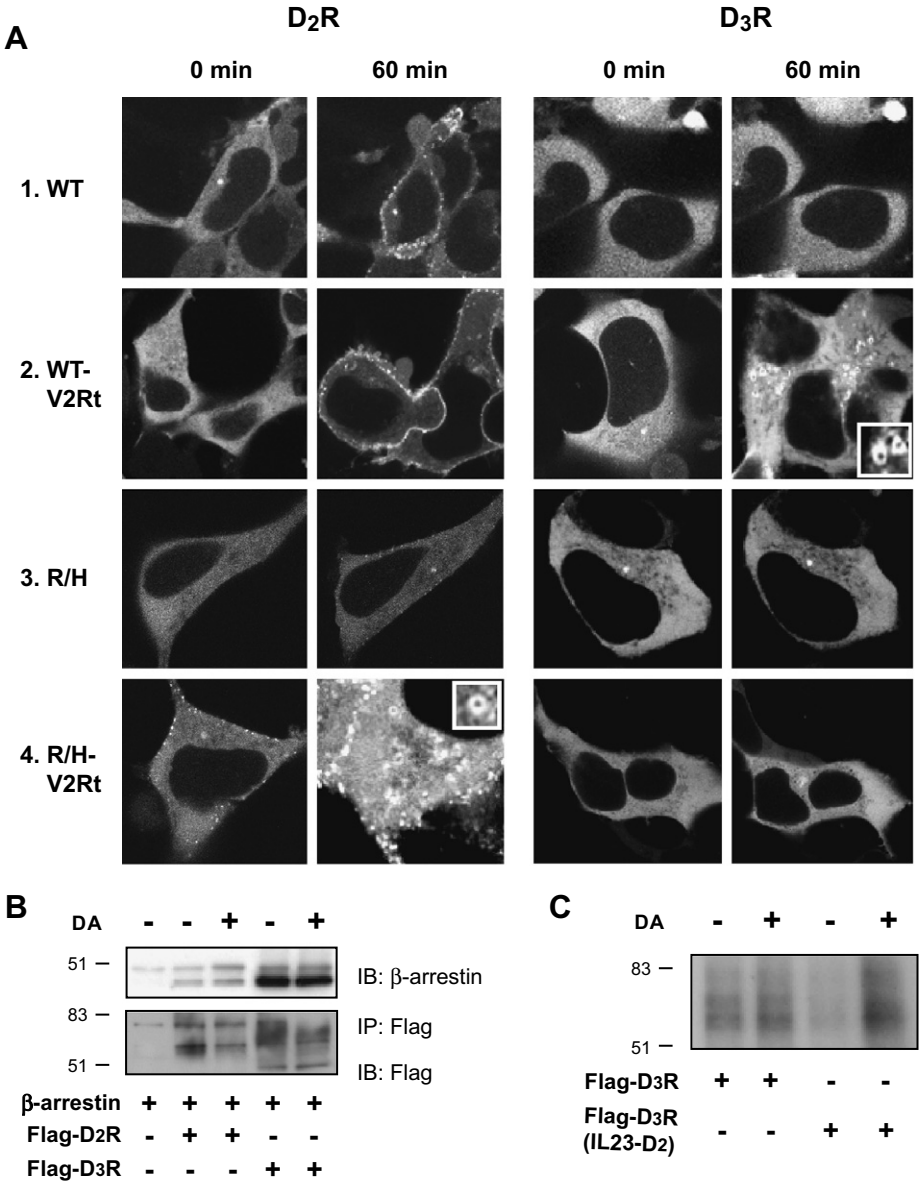

Fig. 1. Effects of the DRY motif and C-terminal tail of the vasopressin type-2 receptor on the intracellular trafficking behavior of D_2R and D_3R. (A) HEK-293 cells were transfected with 3 μg of the corresponding receptor constructs, 2 μg GRK2-pRK5, and 1.5 μg β-arrestin2-GFP per 100-mm culture dish. The cells were stimulated with 10 μM dopamine for 60 min. The receptor expression levels were measured with ^{3}H-spiperone at 3 nM and maintained at ~3 pmol/mg protein. (B) Interaction between β-arrestin and D_2R or D_3R. The cells were transfected with 2 μg Flag-tagged D_2R or D_3R in pCMV5 with 2 μg of β-arrestin2-pCMV5. The cells were stimulated with 10 μM dopamine for 5 min. Immunoprecipitation studies were carried out as described in Materials and methods. The blot was probed with the β-arrestin antibodies, striped, and probed with the M2 Flag antibodies for the immunoprecipitated receptors. The data represents results from five independent experiments. (C) Determination of the receptor regions responsible for the constitutive and agonist-induced phosphorylation of D_3R. The cells were transfected with Flag-tagged D_3R or Flag-tagged D_3–(IL23–D_2), in which the 2nd and 3rd intracellular loops of D_3R had been replaced with those of D_2R. The phosphorylation experiments were carried out as described in Materials and methods. The cells were stimulated with 10 μM dopamine for 5 min and immunoprecipitated with Flag antibodies. The data represents the results from two independent experiments.

The agonist-independent constitutive interaction of β-arrestin with D_2R and D_3R was compared because the intensity of the agonist-dependent dynamic interaction with β-arrestin is not a critical factor to be a class-B GPCR. In contrast to the agonist-induced association between the receptor proteins and β-arrestin, a significantly larger amount of β-arrestin was associated with D_3R than D_2R at the basal state (Fig. 1B). It should be noted that the immunoprecipitated D_2R and D_3R, which are glycosylated proteins, showed some diffuse bands that were not observed in the Mock-transfected group (Fig. 1B, lower panel). The basal phosphorylation of the receptor proteins that would determine the basal interaction with β-arrestin, was higher for D_3R than for D_2R [13] or D_3–(IL23–D_2) (Fig. 1C), in which the 2nd and 3rd intracellular loops were replaced with those of D_2R [19]. This shows that the

stronger interaction with β-arrestin at the basal state, which is presumably caused by the constitutive phosphorylation at the 2nd and 3rd cytoplasmic loops, might be important for determining if GPCRs are class-B.

Roles of DRY motif in the 2nd cytoplasmic loop on the determination of β-arrestin affinity and intracellular trafficking properties of D_2R and D_3R

A structural motif that regulate the basal activation status of receptor trafficking would be important for determining the intracellular trafficking of GPCRs because the constitutive interaction with β-arrestin appears to be a critical factor. The Arg residue in the DRY motif is known to play a key role in the interaction with G protein and β-arrestin, and stabilizes GPCRs in an active or inactivate state [20]. Accordingly, the Arg residue of the DRY motif was mutated to His to determine if the DRY motifs play a role in determining the intracellular trafficking properties of D_2R and D_3R, i.e., class-A or class-B.

$R \rightarrow H$ mutations of the DRY motif decreased agonist-induced β-arrestin translocation markedly (Fig. 1A3). Interestingly, the addition of V_2Rt to the R132H–D_2R caused the constitutive translocation of β-arrestin that could be partially enhanced by the agonist treatment. The long-term agonist treatment of R132H–D_2R–V_2Rt caused the internalization of the receptor proteins, as a complex with β-arrestin to the endocytic vesicles (Fig. 1A4, left panel). On the other hand, the $R \rightarrow H$ mutation of D_3R or D_3R–V_2Rt abolished the β-arrestin translocation (Fig. 1A4, right panel). Considering that a weak β-arrestin translocation was observed with D_3R and D_3R–V_2Rt in the presence of exogenous GRK2, the $R \rightarrow H$ mutations reversed the phenotypes of D_2R–V_2Rt and D_3R–V_2Rt. This suggests that the DRY motif of D_2R stabilizes the receptor conformation at the inactive state so that the D_2R can be fully activated in response to agonist stimulation. Once this restriction is eliminated by a $R \rightarrow H$ mutation, D_2R is released from the inactivating strain, spontaneously activated and shows constitutive desensitization phenotypes upon the addition of V_2Rt [21]. On the other hand, the functional roles of the DRY motif of D_3R appears to stabilize it at the more activated or constitutively desensitized states, which is characterized by constitutive phosphorylation, β-arrestin binding, and low agonist-induced signaling.

$R \rightarrow H$ mutations in the DRY motif abolish agonist-induced signaling and intracellular trafficking of D_2R and D_3R

Since the $R \rightarrow H$ mutation reversed the intracellular trafficking properties of D_2R and D_3R to which V2Rt was attached, the aim was to have a better understanding of the functional roles of the DRY motif. The G protein coupling of D_2R and D_3R were reduced by a $R \rightarrow H$ mutation (Fig. 2A and B). The agonist-induced translocation of β-arrestins to the plasma membrane was significantly lower in R132H–D_2R than in the wild-type (Fig. 2C), which was caused by the inhibition of receptor phosphorylation (Fig. 2D). In accordance with this, the dopamine-induced receptor sequestration (approximately 30% in the wild-type) was completely abolished in the R132H–D_2R (Fig. S2A). Scatchard analysis using radioligand binding studies with ^{3}H-spiperone revealed almost identical K_d and B_{max} values to the wild-type and R132H–D_2R mutant. This suggests that a $R \rightarrow H$ mutation did not have any effect on the binding properties of D_2R. Similar experimental results were also observed with D_3R in that the agonist-induced β-arrestin translocation was abolished by a R128H mutation (Fig. S2B).

These results show that the Arg residues of the DRY motifs of D_2R and D_3R play important roles in the conformational changes for G protein coupling and intracellular trafficking processes that include receptor phosphorylation, β-arrestin translocation, and receptor sequestration.

DRY motif in the 2nd cytoplasmic loop determines the basal affinity for β-arrestin and intracellular trafficking properties of D_2R and D_3R

According to the results shown above, it is conceivable that in order for a GPCR to be class-B, it needs to undergo conformational changes for β-arrestin translocation, and have sufficient binding affinities for β-arrestin. As shown in Fig. 1A2 and A4, D_3R–V_2Rt, which has high affinity for β-arrestin and some G protein coupling, and unexpectedly, R132H–D_2R–V_2Rt, which was not meant to signal, undergo the intracellular trafficking of a class-B GPCR. This suggests that $R \rightarrow H$ might have different effects on the receptor functions depending on the overall conformation.

When the basal affinity for β-arrestin was tested for the receptor constructs used in this study, D_3R showed higher affinity for β-arrestin than D_2R, as shown in Fig. 1B. Moreover, the affinity for β-arrestin increased when the Arg residue mutated to His or V_2Rt was added. Unexpectedly, the G protein coupling of R132H–D_2R which was $<10\%$, increased to as much as 70% of D_2R by the presence of V_2Rt at the C-terminal end (R132H–D_2R–V_2Rt, Fig. 2F).

Discussion

Some GPCRs, including the $AT_{1A}R$ [22–24] and V_2R [25], show G protein-independent and β-arrestin-dependent ERK activation. These new signaling pathways appear to be more evident in the GPCRs that undergo endocytosis with β-arrestin, i.e., type-B GPCRs [26], which depend on their affinity for β-arrestin. Indeed, the importance of the β-arrestin-mediated pathway becomes more important for β_2AR, a type-A GPCR, when its affinity for β-arrestin is increased by exchanging the C-terminal tail with that of V_2R [16].

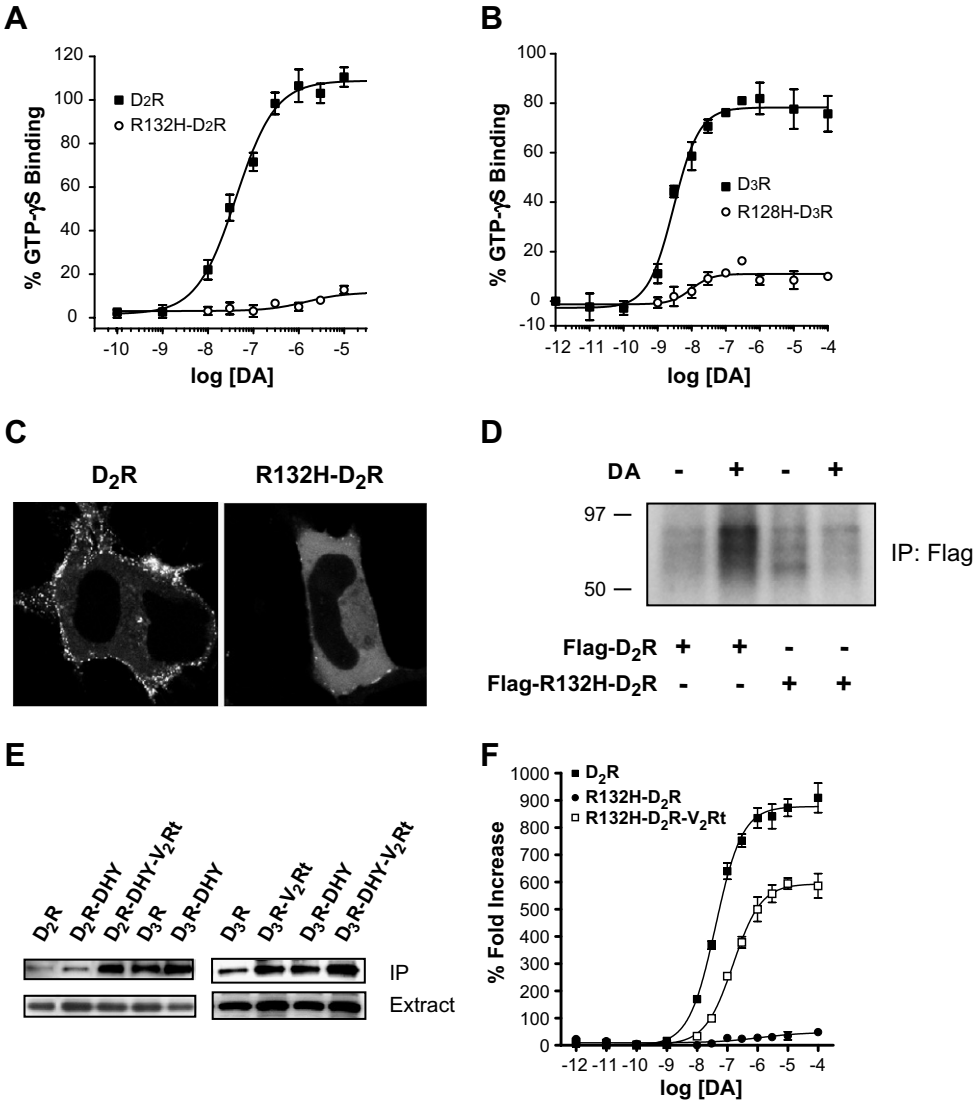

Fig. 2. Effects of R → H mutation and V_2Rt on the G protein coupling and β-arrestin translocation of D_2R and D_3R. (A) HEK-293 cells expressing 1.4 and 1.6 pmol/mg protein of D_2R and R132H–D_2R, respectively, were used. (B) The HEK-293 cells expressing 1.5 pmol/mg protein D_3R and 1.7 pmol/mg protein of R128H–D_3R were transfected with 2 μg Gαo-pCMV5. Equal amounts of the receptor per tube were added. GTP-γS binding was carried out as described in Materials and methods. (C) Effects of the R → H mutation on D_2R-mediated β-arrestin translocation. The cells were transfected with 2 μg D_2R-pCMV5 and 1.5 μg β-arrestin2–GFP (D_2R) or the 2 μg R132H–D2R, 2 μg GRK2–pRK5, and 1.5 μg β-arrestin2–GFP (R132H–D_2R). The cells were stimulated with 10 μM DA for 5 min. The receptor expression level was ~3 pmol/mg protein. (D) Effects of a R → H mutation on receptor phosphorylation. The HEK-293 cells were transfected with 3 μg Flag-D_2R or Flag-tagged R132H–D_2R. The data represents the results from three independent experiments. (E) Interaction between the different receptor mutants and β-arrestin. The cells were transfected with the corresponding receptor cDNA and 2 μg β-arrestin2-pCMV5. The receptor expression levels were ~3 pmol/mg protein. The data represents the results from two independent experiments. (F) Comparison of GTP-γS binding to D_2R, R132H, and R132H–D_2R–V_2Rt. The expression levels were adjusted to ~1.5 pmol/mg protein.

In this study, D_3R but not D_2R was converted to a class-B GPCR when V_2Rt was attached to their ends. Because the C-terminal tails of D_2R and D_3R are similar, the Arg residues of the DRY motifs in these receptors appear to play important roles in β-arrestin binding as well as in maintaining the receptor conformations. As shown in Fig. 2E, the basal β-arrestin binding of D_2R was low but was enhanced by the R → H mutation. Moreover, the addition of V_2Rt, resulted in the constitutive translocation of β-arrestin (R132H–D_2R–V_2Rt), which is a characteristic of constitutively desensitized GPCR [27,28]. This indicates that the conformation of R132H–D_2R is stabilized to a desensitized state, which suggests that the Arg residue in this DRY motif stabilizes D_2R in the inactive state to allow full activation when stimulated with an agonist. In contrast, the basal binding of D_3R to β-arrestin was already high with the R → H mutation having little effect. This indicates that the serine/threonine clusters in the C-terminus tail might provide the main driving force for the stable interaction between the receptor and β-arrestin, and the DRY motif might work as a switch to determine their intracellular trafficking behavior.

In conclusion, for a GPCR to be a class-B, it needs to undergo agonist-induced conformational changes. The DRY motif and C-terminus tail have complementary roles, and G protein coupling is determined by the overall receptor conformation rather than the specific primary sequences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.055.

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