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RESEARCH PAPER

The N-terminal region of the dopamine D_2 receptor, a rhodopsin-like GPCR, regulates correct integration into the plasma membrane and endocytic routes

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BACKGROUND AND PURPOSE
Functional roles of the N-terminal region of rhodopsin-like GPCR family remain unclear. Using dopamine D_2 and D_3 receptors as a model system, we probed the roles of the N-terminal region in the signalling, intracellular trafficking of receptor proteins, and explored the critical factors that determine the functionality of the N-terminal region.

EXPERIMENTAL APPROACH
The N-terminal region of the D_2 receptor was gradually shortened or switched with that of the D_3 receptor or a non-specific sequence (FLAG), or potential N-terminal glycosylation sites were mutated. Effects of these manipulations on surface expression, internalization, post-endocytic behaviours and signalling were determined.

KEY RESULTS
Shortening the N-terminal region of the D_2 receptor enhanced receptor internalization and impaired surface expression and signalling; ligand binding, desensitization and down-regulation were not affected but their association with a particular microdomain, caveolae, was disrupted. Replacement of critical residues within the N-terminal region with the FLAG epitope failed to restore surface expression but partially restored the altered internalization and signalling. When the N-terminal regions were switched between D_2 and D_3 receptors, cell surface expression pattern of each receptor was switched. Mutations of potential N-terminal glycosylation sites inhibited surface expression but enhanced internalization of D_2 receptors.

CONCLUSIONS AND IMPLICATIONS
Shortening of N-terminus or mutation of glycosylation sites located within the N-terminus enhanced receptor internalization but impaired the surface expression of D_2 receptors. The N-terminal region of the D_2 receptor, in a sequence-specific manner, controls the receptor's conformation and integration into the plasma membrane, which determine its subcellular localization, intracellular trafficking and signalling properties.

Abbreviations
DA, dopamine; NT, N-terminus; PMA, phorbol myristate acetate; TM, transmembrane domain

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Introduction

GPCRs are classified into rhodopsin-like, secretin-like and metabotropic glutamate-like families based on the similarities of amino acid sequences in the N-terminal region (Bockaert and Pin, 1999). Rhodopsin-like GPCRs are further classified into subfamilies according to their ligands, which include catecholamines, peptides and glycoprotein hormones. Dopamine D_2 and D_3 receptors belong to the catecholamine subfamily and bind to dopamine, a small molecule that presumably binds in a cavity formed by transmembrane domains (TMs) III to VI (Dixon *et al.*, 1987; Strader *et al.*, 1987; Chien *et al.*, 2010). D_2 and D_3 receptors are important targets for the treatment of various diseases associated with motor, emotional and endocrine dysfunction as well as drug addiction, which are caused by improper regulation of dopaminergic transmission (Thomas *et al.*, 2008; Cho *et al.*, 2010a).

Like other members in this family of GPCRs, D_2 and D_3 receptors contain several highly conserved amino acids: an aspartic acid residue in the second TM of GPCRs is important for agonist binding (Neve *et al.*, 1991) and/or G-protein coupling (Ceresa and Limbird, 1994); two highly conserved cysteine residues within the first and second extracellular loops that form a disulfide link that probably stabilizes the GPCRs in certain conformations (Dohlman *et al.*, 1990; Savarese *et al.*, 1992); the Asp-Arg-Tyr (DRY) motif, which is located at the boundary between TM III and intracellular loop 2, controls G-protein coupling and intracellular trafficking (Kim *et al.*, 2008); and the NPX(X)Y motif in the seventh TM is involved in the recruitment of adaptor proteins and clathrin for endocytosis (Barak *et al.*, 1995). Also a number of GPCRs including D_2 and D_3 receptors contain potential glycosylation sites in the extracellular regions (Clagett-Dame and McKelvy, 1989; Jarvie and Niznik, 1989; David *et al.*, 1993).

Although the TM regions are relatively well-conserved among GPCRs, most GPCRs, including D_2 and D_3 receptors, differ in their amino acid sequences and lengths of the N-terminal region and intracellular loops (Giros *et al.*, 1990). Each of these domains provides specific and characteristic properties to these various receptor proteins. The roles of the intracellular loops of D_2 and D_3 receptors have been extensively characterized in terms of G-protein coupling, intracellular trafficking and protein interactions (Kim *et al.*, 2001; Kim *et al.*, 2008). On the other hand, the roles of the N-terminal region have not been reported for the catecholamine receptors in terms of their involvement in various receptor functions and behaviours, such as ligand binding, subcellular localization, intracellular trafficking and signalling.

The crystal structure of the D_3 receptor was recently reported (Chien *et al.*, 2010), and a homology model of the D_2 receptor was created based on the structural information from D_3 receptors. The three-dimensional structure around the TM domains was clearly shown, and the ionic interactions among the well-conserved amino acid residues on the TM regions and adjacent submolecular domains were well documented. On the other hand, the structural features of the N-terminal region could not be determined because the N-terminal residues did not have enough density to interpret.

In this study, we created deletion mutants in the N-terminal region of the D_2 receptor, chimeric receptors between D_2 and D_3 receptors in which their N-terminal regions were exchanged and point mutants in the potential glycosylation sites. A battery of assays was then performed for these mutant receptors including cell surface expression, ligand binding, signalling, internalization and desensitization. Our results show that the N-terminal regions of D_2 receptors determine the subcellular localization of receptor proteins in a sequence-specific manner in which potential glycosylation sites play critical roles. It is postulated that the N-linked glycosylation sites in the terminus of D_2 receptors are necessary for the integration of receptor proteins into the plasma membrane and for maintaining a stable conformation.

Methods

Materials

Human embryonic kidney cells (HEK-293) were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell culture media and fetal bovine serum were obtained from Hyclone laboratories Inc. (Logan, UT, USA). DA, quinpirole, sulpiride, haloperidol, phorbol myristate acetate (PMA), forskolin, sucrose and antibodies to haemagglutinin (HA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]-Sulpiride (84 Ci·mmol^{-1}) and [^{3}H]-spiperone (25.5 Ci·mmol^{-1}) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Antibodies to actin, caveolin-1 and GRK2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to β-arrestins were provided by Dr Lefkowitz (Duke University).

Plasmid constructs

Wild-type (WT) D_2 and D_3 receptors in pCMV5 and pEGFP-N1 were as described in a previous study (Kim *et al.*, 2001). N-terminal deletion mutants of D_2 receptors (Δ(2–10), Δ(2–22), Δ(2–34) were prepared in pcDNA 3.1 Zeo (+), pCMV5 and pEGFP-N1. All of them were tagged with FLAG sequences at the N-terminus (NT). The chimeric receptors between D_2 and D_3 receptors, in which the N-terminal regions were exchanged, were produced as described in the following discussion. Firstly, the human D_2 and D_3 receptors were subcloned into the *Saccharomyces*/*E. coli* phagemid vector pRS314 (pRS314-D_2R and pRS314-D_3R). The N-terminal fragment of D_2 receptors was amplified using a primer composed of the sequence from pRS314 and the first 28 nucleotides of D_2 receptors (oligo-1 in Table 1) and another primer composed of the sequence from the first TM domain of D_3 receptors and the sequence from the distal part of the D_2 receptor-NT (oligo-2 in Table 1). As in D_2 receptors, the N-terminal region of D_3 receptors was amplified using one primer composed of the sequence from pRS314 and the first 28 nucleotides of D_3 receptors (oligo-3 in Table 1), another primer composed of the sequence from the first TM domain of D_2 receptors and the sequence from the distal part of the D_3 receptor NT (oligo-4 in Table 1). Each DNA fragment was transfected into *Saccharomyces cerevisiae* together with pRS314-D_2 receptors or pRS314-D_3 receptors cut with *Sac II* and *Not I*. Recombinant clones were selected based on tryptophan auxotrophy and the yeast plasmids were isolated. The yeast D_2 receptor plasmid containing the NT of D_3 receptors,

Table 1
Oligos used to create NT chimeric receptors between D_2 receptors and D_3 receptors

Oligo-1: GTAATACGACTCACTATAGGGCGAATTGGAGCATGGAT CCACTGAATCTGTCCTGGTATG
Oligo-2: GATGAGCGCGCAGTAGGAGAGGGCATAGTAGTTGTAG TGGGGTCTGTCC
Oligo-3: GTAATACGACTCACTATAGGGCGAATTGGAGCATGGCA TCTCTGAGTCAGCTGAGTAGCC
Oligo-4: GCGATGAGCAGGGTGAGCAGTGTGGCATAGTAGGCA TGTGGGCGGGCC

D_2 receptor-(D_3-NT) and the yeast D_3 receptor plasmid containing the NT of D_2 receptors, D_3 receptor-(D_2 receptor-NT) were amplified with alternate oligos designed for the NT and C-terminus of D_2 and D_3 receptors. They were then subcloned into the *Hind III*/*Xba I* sites of the pCDNA3.1 Zeo(+) vector and were also subcloned into pEGFP-N1. Chimeric receptors between D_2 and D_3 receptors in which the second and third intracellular loops were exchanged, were as described in previous studies (Robinson and Caron, 1996; Min *et al.*, 2011). The mutants in the potential N-linked glycosylation sites (GlyX) of WT-D_2 receptors and D_2 receptor-D_3NT were created by altering three asparagine residues at the 5th, 17th and 23rd position of D_2NT as well as at the 12th and 19th position of D_3NT (Figure 1A) to the glutamine residues. Cells were transiently transfected by the calcium phosphate method unless specified otherwise.

Small hairpin RNAs of β-arrestins and GRK2

HEK-293 cells stably expressing shRNA constructs of the scrambled sequence, β-arrestin1/β-arrestin2 or GRK2 were developed. Levels of endogenous β-arrestins and GRK2 were detected by immunoblotting. The shRNA plasmids were obtained as published previously (Zhang *et al.*, 2008; Cho *et al.*, 2010b).

Determination of ligand-binding properties

Cells expressing D_2 or D_3 receptors were incubated with 2.2 or 7.2 nM [^{3}H]-sulpiride and increasing concentrations of dopamine or quinpirole, respectively, for 150 min at 4°C. Cells were washed three times with ice-cold serum-free Minimum Essential Medium (MEM) containing 10 mM HEPES, pH 7.4, lysed with 1% SDS, and counted with liquid scintillation counter.

Internalization assay

The internalization of D_2 and D_3 receptors was measured based on the hydrophilic properties of [^{3}H]-sulpiride (Kim *et al.*, 2001). HEK-293 cells expressing D_2 receptors were seeded 1 day after transfection at a density of 1.5×10^5 cells per well in 24-well plates. The following day, cells were rinsed once and pre-incubated with 0.5 mL of pre-warmed, serum-free medium containing 10 mM HEPES, pH 7.4 for 15 min at 37°C. Cells were stimulated with 10 μM of DA or 1 μM of PMA for 0–120 min as indicated. Cells were incubated with 250 μL of [^{3}H]-sulpiride (final concentration 2.2 nM for D_2 receptors and 7.2 nM for D_3 receptors) at 4°C for 150 min in the absence and presence of unlabelled competitive inhibitor (10 μM haloperidol). Cells were washed and then lysed with 1% SDS and the remaining radioactivity was counted with a liquid scintillation counter.

Measurement of D_2 and D_3 receptors in intracellular and extracellular regions

The ratio of intracellular/total receptor levels was calculated as described previously (Kim *et al.*, 2001). Total receptor level was assessed using [^{3}H]-spiperone (final concentration, 3 nM), a hydrophobic ligand capable of labelling both the intra- and extracellular components of the receptor. Intracellular receptor levels were measured by displacing the extracellular binding with a high concentration of the hydrophilic ligand, sulpiride (final concentration, 3 μM). Non-specific binding was determined in the presence of 10 μM haloperidol. Binding reactions were determined after an incubation period of 3 h at 14°C to prevent receptor recycling and were terminated by washing three times with vacuum filtration over Whatman GF/C glass-fibre filters using ice-cold wash buffer (Whatman, Dassel, Germany). Samples were mixed with 2 mL Optiphase Supermix and counted with a Wallac 1450 Microbeta scintillation counter (PerkinElmer, MA, USA).

Immunoprecipitation

After 48 h transfection, the cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 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 50% slurry of anti-FLAG-agarose beads (Sigma/Aldrich) for 2–3 h on the rotation wheel. The beads were washed with washing buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 10% glycerol, 1% NP-40) three times for 10 min each. The immunoprecipitates were analysed by immunoblotting.

Whole cell cAMP assays

Cellular cAMP was measured by an indirect reporter gene method (Himmler *et al.*, 1993; Hearn *et al.*, 2002). A reporter plasmid containing the firefly luciferase gene under the control of multiple cAMP responsive elements (CREs) and with pRL-TK control vector was utilized. Transfected cells were seeded in 24-well plates and each transfection set was organized into three identical groups. The cells were treated with 2 μM forskolin and quinpirole (10^{-12}–10^{-8} M) for 4 h and harvested; the relative luciferase expression was measured using the dual luciferase assay kit (Promega, Madison, WI, USA). Data were normalized by expressing cAMP levels as a percentage of the forskolin-stimulated cAMP for each experiment. Dose–response curves were fitted with GraphPad Prism (GraphPad, San Diego, CA).

Immunocytochemistry and confocal microscopy

One day after transfection, the cells were seeded onto 35 mm dishes containing a centred, 1-cm well that was formed from a glass coverslip-sealed hole in plastic (confocal dishes) and allowed to recover for one day. Next day, the cells were fixed

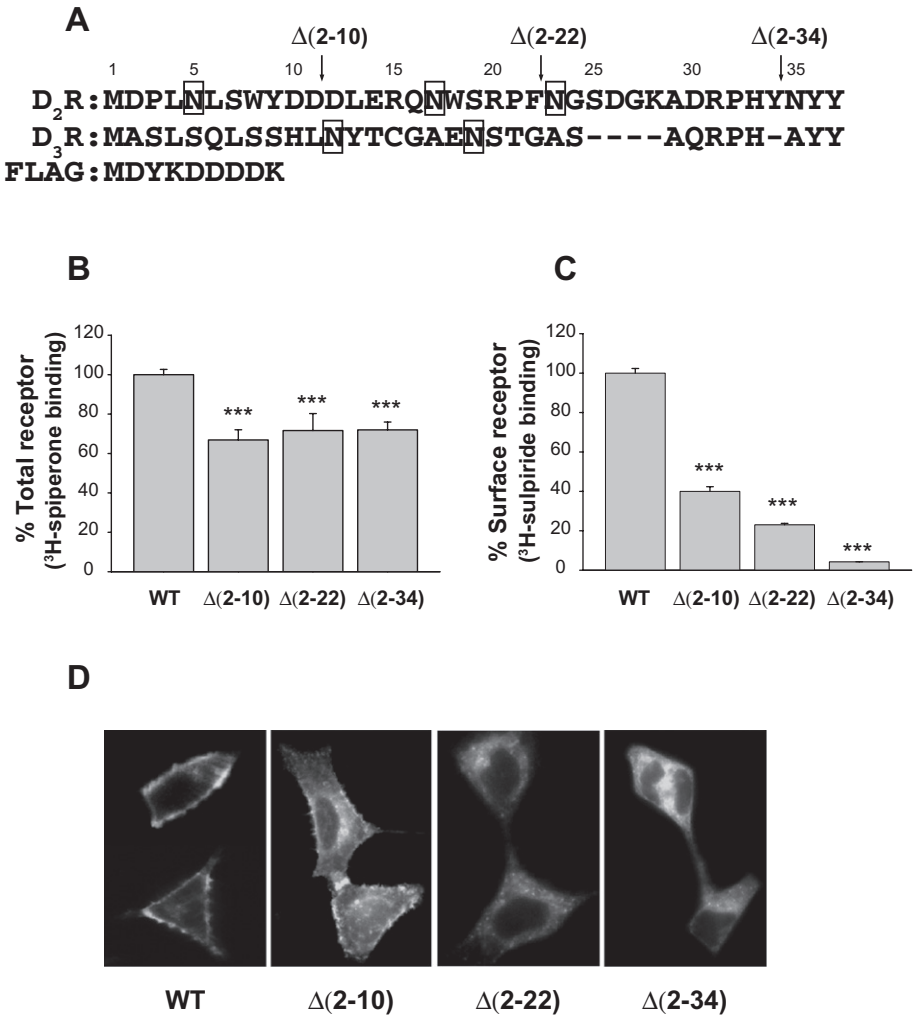

Figure 1

Effects of shortening the N-terminal region on the surface expression of the dopamine D_2 receptor (D_2R). (A) Alignment of the amino acid sequences within the N-terminal regions of D_2R and D_3R. Vertical arrows show the positions within the N-terminal tail where deletions were made. The potential glycosylation sites of D_2R and D_3R are shown in square boxes. (B, C) Effects of shortening the N-terminal region on the cell surface expression of D_2R. HEK-293 cells were transfected with 2 μg of each construct in pCMV5 per 100 mm culture dishes. Cells were treated with 3 nM [^{3}H]-spiperone (for total binding, cell surface and hydrophobic interior) or 2.2 nM [^{3}H]-sulpiride (for cell surface binding) dissolved in serum-free media for 1 h at room temperature in the presence and absence of 10 μM haloperidol. Cells were washed with ice-cold, serum-free media three times, dissolved with 1% SDS and then counted with a liquid scintillation counter. ***$P < 0.001$ compared with WT group. (D) Microscopic images of N-terminal deletion mutants of D_2R. Cells were transfected with constructs in pEGFP-N1 and observed 48–50 h later with a Nikon Ti2000 live cell imaging system.

with 4% paraformaldehyde, for 15 min at room temperature and permeabilized with 0.25% Triton X-100. Cells were labelled with antibodies to caveolin-1 at a 1:1000 dilution and with Alexa 594-conjugated anti-mouse antibodies at a 1:500 dilution. The cells were examined with a Nikon Ti2000 live cell imaging system (Nikon, Tokyo, Japan) or a laser scanning confocal microscope (TCS SP5/ABOS/Tadem, Leica, Heidelberg, Germany).

Statistics

All of the results are expressed as means ± SEM. Comparisons between groups were performed using ANOVA. For some results, Student's *t*-test was also used.

Results

N-terminal region affects the cell surface expression of D_2 receptors

The recently reported and postulated crystal structures of D_3 and D_2 receptors showed that the N-terminal region is extracellular and is attached to the first TM with a high degree of flexibility (Chien *et al.*, 2010). Along with the intracellular loops, the N-terminal regions of GPCRs are highly variable in sequence homology. For example, the sequence homology between D_2 and D_3 receptors is high with the two having 46% overall amino acid homology and 78% identity in the TMs (Giros *et al.*, 1990); however, the alignment of amino acid

sequences within the N-terminal regions of D_2 and D_3 receptors shows that they are disparate in composition, except for several amino acid residues near the first TM (Figure 1A). The N-terminal region deletion mutants, Δ(2–10)-, Δ(2–22)-, and Δ(2–34)-D_2 receptor, represent mutants in which 9, 21 and 33 amino acids located after the first methionine residue were deleted, respectively (Figure 1A). The expression profiles of the D_2 receptor deletion mutants were determined by [^{3}H]-spiperone binding, which detects D_2 receptors expressed both on the cell surface and intracellularly. When equal amounts of deletion mutants in the same mammalian expression vector and cloning sites were transfected, the expression levels of the N-terminal deletion mutants were around 65% of WT-D_2 receptors (Figure 1B). Expression of these mutants on the plasma membrane was measured by binding of [^{3}H]-sulpiride, which is highly hydrophilic. The [^{3}H]-sulpiride binding was about 40% for Δ(2–10)-D_2 receptors, 23% for Δ(2–22)-D_2 receptors and almost undetectable for Δ(2–34)-D_2 receptors compared with WT-D_2 receptors (Figure 1C). These results were confirmed by microscopic studies (Figure 1D). More receptor proteins were observed inside the cells as the N-terminal region of the D_2 receptor was shortened.

Shortening of the N-terminal region impairs the signalling of D_2 receptors

Next, the ligand-binding and signalling properties of deletion mutants were examined by determining the competition between radiolabelled antagonist and agonist. The surface expression of the deletion mutants was matched by transfecting the DNAs of WT-, Δ(2–10)-, Δ(2–22)-D_2 receptors in the ratio of 1:3:7. As shown in Figure 2A, the [^{3}H]-sulpiride/dopamine competition binding properties of Δ(2–10)-D_2 and Δ(2–22)-D_2 receptors were similar to those of WT-D_2 receptors. The surface expression of Δ(2–34)-D_2 receptors was too low to conduct reliable studies. These results confirm that the N-terminal region of D_2 receptors, which belongs to the catecholamine subgroup of the rhodopsin GPCR family, does not affect ligand binding.

The signalling of D_2 receptors, which was measured by the inhibition of cAMP production, gradually decreased as the N-terminal region was shortened under experimental conditions in which receptor expression levels of each D_2 receptor construct on the cell surface were adjusted to be equal (Figure 2B). The EC_{50} values obtained from WT-, Δ(2–10)- and Δ(2–22)-D_2 receptors were 69, 243 and 796 pM, respectively. Desensitization is defined as the attenuation of receptor responsiveness by prolonged or previous exposure to an agonist. Desensitization of D_2 receptors, which is known to involve receptor phosphorylation, was induced by pretreatment with dopamine. It is known that agonist-induced (homologous) desensitization does not occur with D_2 receptors (Westrich and Kuzhikandathi, 2007; Cho *et al.*, 2010b) and this property was not altered when the N-terminal region was shortened (Figure 2C).

N-terminal region is involved in the regulation of the intracellular trafficking of D_2 receptors

The internalization of GPCRs is mutually related to the signalling. Since the signalling of D_2 receptors was altered when the N-terminal region was shortened, the effects on internalization were also examined. As shown in Figure 3A, the internalization rate of D_2 receptors proportionally increased as the N-terminal region was further shortened. Microscopic images taken from cells treated with dopamine for 1 h also revealed that a larger fraction of Δ(2–22)-D_2 receptors was observed in the cytoplasmic region compared with WT-D_2 receptors (Figure 3B). Interestingly, the vesicles of Δ(2–22)-D_2 receptors were smaller than those of the WT-D_2 receptors. The net increase in the internalization of D_2 receptors could be caused either by an increase in the internalization rate or by a decrease in the return rate of the internalized receptor. The return of internalized GPCRs is determined by the recycling rate and down-regulation of internalized receptors. To assess the recycling of internalized D_2 receptors, internalization was induced by treating cells with 10 μM dopamine for 1 h; the cells were then washed and the changes in receptor numbers on the cell surface were determined at various time points. As shown in Figure 3C, the percentage of returning Δ(2–22)-D_2 receptors to the plasma membrane was similar to that of WT-D_2 receptors. In addition, no difference in the down-regulation of receptors was observed between WT- and Δ(2–22)-D_2 receptors after long-term treatment with the agonist (12 and 24 h, Figure 3D), suggesting that the internalized receptor eventually returned to the plasma membrane without degradation. These results suggest that the increased internalization of N-terminal deletion mutants of the D_2 receptor is caused by an increase in the endocytosis rate.

N-terminal deletion impairs the signalling efficiency but increases the internalization of D_2 receptors

As shown in Figures 2B and 3A, the signalling efficiencies decreased and the receptor internalization increased when the N-terminal region of D_2 receptors was shortened. To determine whether decreases in the signalling efficiencies of the N-terminal region mutants were caused by increases in receptor internalization, the internalization of D_2 receptors was inhibited by sucrose treatment (Figure 4A), which is routinely used to block the internalization of GPCRs (Daukas and Zigmond, 1985; Cho *et al.*, 2010b). Compared with the WT-D_2 receptors, the extent of inhibition of receptor internalization by sucrose treatment was smaller for Δ(2–10) and Δ(2–22) D_2 receptors (82.3 vs. 65.0, and 48.4%, respectively), suggesting that additional endocytic pathways with distinct characteristics could be involved in the endocytosis of Δ(2–10) and Δ(2–22) D_2 receptors. The signalling of WT-D_2 receptors was not influenced by sucrose treatment (Figure 4B), but the dose–response curves of Δ(2–10)-D_2 receptors (Figure 4C) and Δ(2–22)-D_2 receptors (Figure 4D) shifted to the left, suggesting that decreases in the signalling efficiencies of N-terminal region mutants could be caused by the large increase in receptor internalization.

N-terminal region controls the internalization of D_2 receptors independently of GRK2 and β-arrestins

The internalization of GPCRs involves multiple cellular processes, including receptor phosphorylation and recruitment of endocytic machinery. GRK2 and β-arrestins are two critical

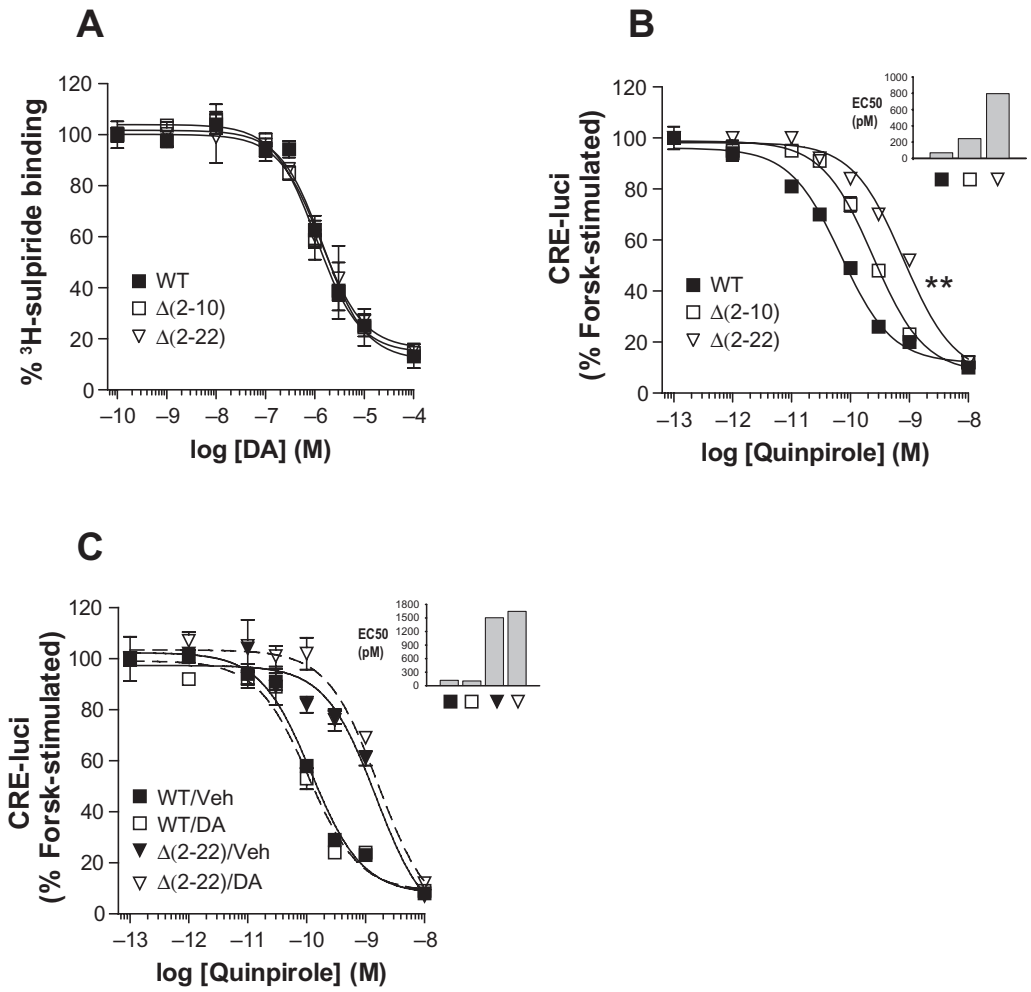

Figure 2

Roles of the N-terminal region in signalling of the dopamine D_2 receptor (D_2R). (A) Effects of shortening the N-terminal region on the ligand-binding properties of D_2R. Cells transfected with WT-, Δ(2–10)- or Δ(2–22)-D_2R were treated with 2.2 nM [^{3}H]-sulpiride and increasing concentrations of dopamine (DA) for 1 h at room temperature in the presence and absence of 10 μM haloperidol. (B) Effects of shortening the N-terminal region on the signalling of D_2R. HEK-293 cells were transfected with WT-, Δ(2–10)- or Δ(2–22)-D_2R and their cell surface expression was adjusted to ~0.5 pmol·mg^{-1} protein. Cellular cAMP levels were measured as described in the Methods section. $^{**}P < 0.01$ when the Δ(2–22)-D_2R curve was compared with the WT-D_2R curve. (C) Effects of shortening the N-terminal region on the desensitization of D_2R. Cells expressing WT- or Δ(2–22)-D_2R were pretreated with 10 μM dopamine for 10 min and washed with serum-free media, and then dose–response curves were determined with increasing concentrations of quinpirole. Results are representative of three independent experiments.

cellular elements that mediate the internalization of D_2 receptors through a clathrin-dependent endocytic pathway. To test whether GRK2 or β-arrestins are involved in the enhancement of the internalization of the D_2 receptors with shortened N-terminal regions, endogenous GRK2 or β-arrestin1 and 2 were knocked-down through stable expression of shRNAs. As shown in Supporting Information Figure S1A–D, knockdown of GRK2 or β-arrestin1/2 moderately inhibited the internalization of WT-D_2 receptors, Δ(2–10)- and Δ(2–22)-D_2 receptors in parallel. The interaction with GRK2 or β-arrestin2, which determines the endocytic properties of GPCRs, was similar in WT- and Δ(2–22)-D_2 receptors (Supporting Information Figure S1E and F). These results suggest that increases in the internalization of Δ(2–10)- and Δ(2–22)-D_2 receptors occur independently of GRK2 and β-arrestins.

N-terminal region determines the localization of D_2 receptors in specific plasma membrane microdomains

Clathrin-coated pits and caveolae are two representative plasma membrane microdomains where the endocytosis of GPCRs occurs. To better understand the molecular mechanisms involved in the increased internalization of N-terminal deletion mutants of D_2 receptors, their associations with these two plasma membrane microdomains were examined. Epsin is known to contribute to clathrin-mediated endocytosis by binding to phospholipids, clathrin and the AP2 complex (Wendland, 2002). Methyl-β-cyclodextrin (MβCD) causes depletion of cholesterol in caveolae and has been used as a cholesterol depleting-agent. The internalization of WT-D_2

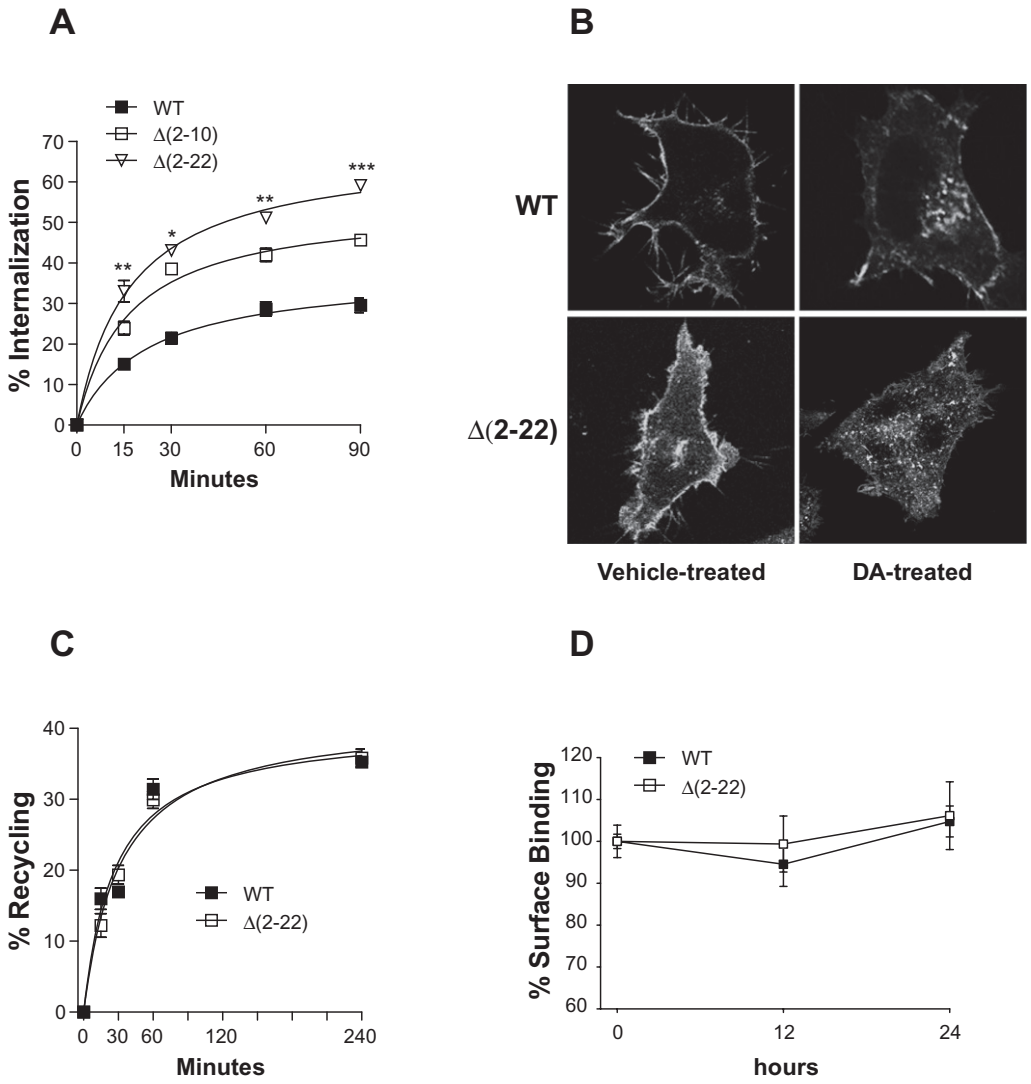

Figure 3

Effects of shortening the N-terminal region on the intracellular trafficking of the dopamine D_2 receptor (D_2R). (A) Effects of shortening the N-terminal region on the time course of agonist-induced D_2R internalization. The expression levels of each construct were adjusted to ~0.5 pmol·mg^{-1} protein. Cells were treated with 10 μM dopamine (DA) for 15, 30, 60 and 90 min, and the internalization assay was conducted as described in the Methods section. Comparisons of the internalization among three experimental groups were performed using ANOVA at each time point. The magnitude of internalization of the Δ(2–22) group was significantly different from that of WT group ($P < 0.001$) at all time points. The magnitude of internalization of Δ(2–10) group was significantly different than that of WT group: $P < 0.01$ at 15 and 60 min; $P < 0.001$ at 30 and 90 min. *, **, *** represents $P < 0.05$, 0.01, and 0.001, respectively, when the Δ(2–10) group and Δ(2–22) group was compared. (B) Comparison of the microscopic images of WT- and Δ(2–22)-D_2R before and after treatment with 10 μM dopamine for 1 h. Cells were transfected with GFP-tagged WT- or Δ(2–22)-D_2R and treated with 10 μM dopamine for 1 h. (C) Effects of shortening the N-terminal region on the recycling of internalized D_2R. Cells transfected with the corresponding construct were treated with 50 μg·mL^{-1} cyclohexamide for 1 h, followed by treatment with 10 μM dopamine for 1 h and then washed with serum-free media for 15, 30, 60 min and 4 h at 37°C. Cells were incubated with 2.2 nM [^{3}H]-sulpiride for 150 min at 4°C to measure the remaining D_2R on the cell surface. The dopamine-induced internalization was 35 and 47% for WT- and Δ(2–22)-D_2R, respectively. (D) Effects of shortening the N-terminal region on the down-regulation of D_2R. Cells transfected with the corresponding construct were treated with 50 μg·mL^{-1} cyclohexamide for 1 h, followed by 1 μM quinpirole for 12 or 24 h, washed with serum-free media at 4°C, and then incubated with 2.2 nM [^{3}H]-sulpiride for 150 min at 4°C. Results are representative of three independent experiments.

receptors was inhibited by treatment with 3 mM of MβCD, and by co-expression of epsin (204–458), a dominant negative of epsin (Figure 5A and B). In contrast to WT-D_2 receptors, the internalization of Δ(2–22)-D_2 receptors was insensitive to treatment with MβCD (Figure 5A) but was sensitive to co-expression of epsin (204–458) (Figure 5B). These results suggest that the endocytic pathways of D_2 receptors could have been altered when the N-terminal region was shortened. To test whether the interactions with clathrin or caveolin-1 were altered by shortening of the N-terminal

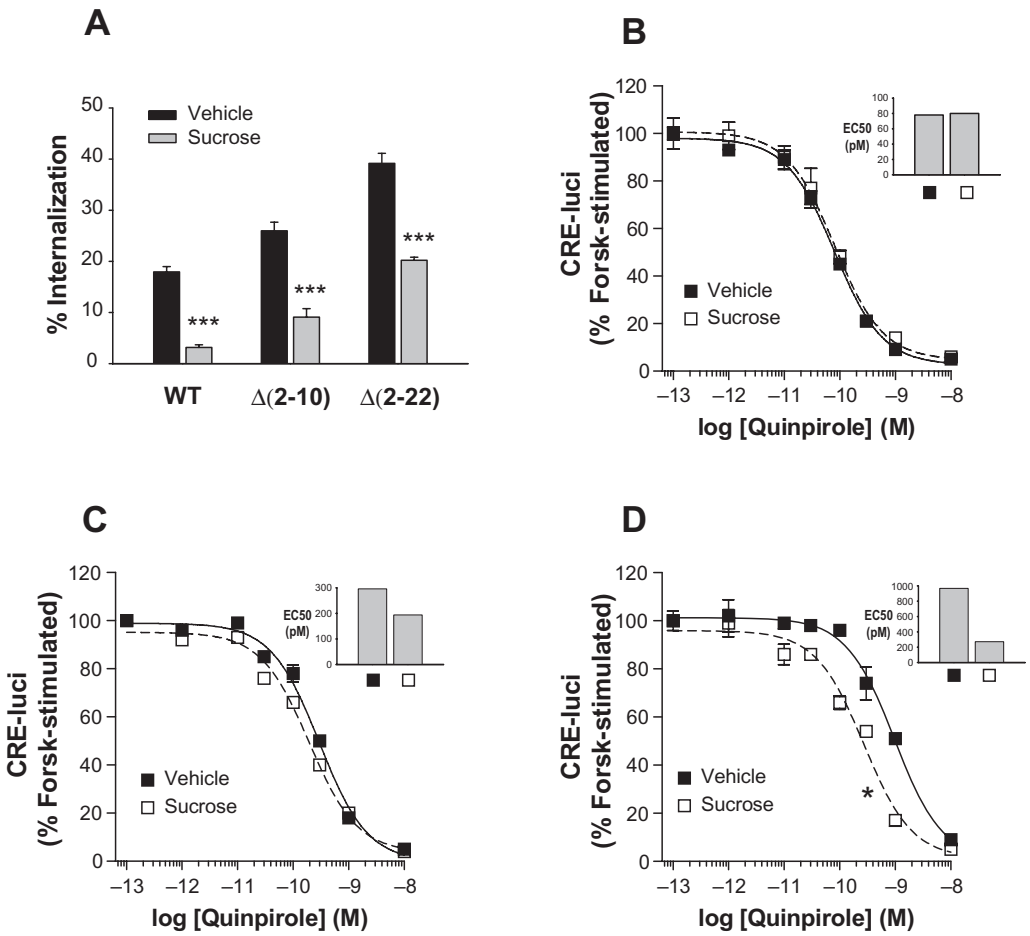

Figure 4

Relationship between receptor internalization and signalling of N-terminal deletion mutants (A) Effects of sucrose treatment on agonist-induced internalization of WT and N-terminal deletion mutants of D_2R. Cells were treated with either vehicle or 0.45 M sucrose for 20 min, followed by treatment with 10 μM dopamine (DA) for 1 h. Internalization assay was conducted as described in the Methods section. ***$P < 0.001$ compared with vehicle group. (B) Effects of inhibition of internalization on the signalling of WT-D_2R. Cells were treated with sucrose as in (A) and dose–response curves were obtained for WT-D_2R. (C) Effects of inhibition of internalization on the signalling of Δ(2–10)-D_2R. (D) Effects of inhibition of internalization on the signalling of Δ(2–22)-D_2R. *$P < 0.05$ compared with vehicle group. Results are representative of three independent experiments.

region, immunoprecipitations were conducted. In agreement with internalization results, the interactions between caveolin-1 (Figure 5C) and WT-D_2 receptors, but not Δ(2–22)-D_2 receptors, were increased when cells were stimulated with agonist. These results were also confirmed by immunocytochemistry. WT-D_2 receptors and caveolin-1 were located on the plasma membrane in the resting state (Supporting Information Figure S2A). Some of these proteins moved into the cytosolic region in response to agonist stimulation and co-localized in the endocytic vesicles (Supporting Information Figure S2B). On the other hand, co-localization between Δ(2–22)-D_2 receptors and caveolin-1 was less prominent and tended to exclude each other in the endocytic vesicles (Supporting Information Figure S2C and D). With regard to their interaction with clathrin, both WT-D_2 receptors and Δ(2–22)-D_2 receptors showed a similar pattern of activity (Figure 5D). These results show that the WT-D_2 receptor follows two endocytic pathways that include clathrin-coated pits and caveolae. These properties were altered when the N-terminal region was shortened, suggesting that an intact NT is needed for correct intracellular trafficking of D_2 receptors.

The length and amino acid sequence of the N-terminal region could contribute to the regulation of D_2 receptor functions

The signalling and intracellular trafficking of D_2 and D_3 receptors are determined by the sequence of amino acids within the second and third intracellular loops (Robinson and Caron, 1996; Kim *et al.*, 2001). Because the shortening of the N-terminal region also affected the signalling and internalization of the D_2 receptor (Figures 2 and 3), we questioned whether the specific amino acid sequence or the relative length of the N-terminal region is a critical factor that determines these functions.

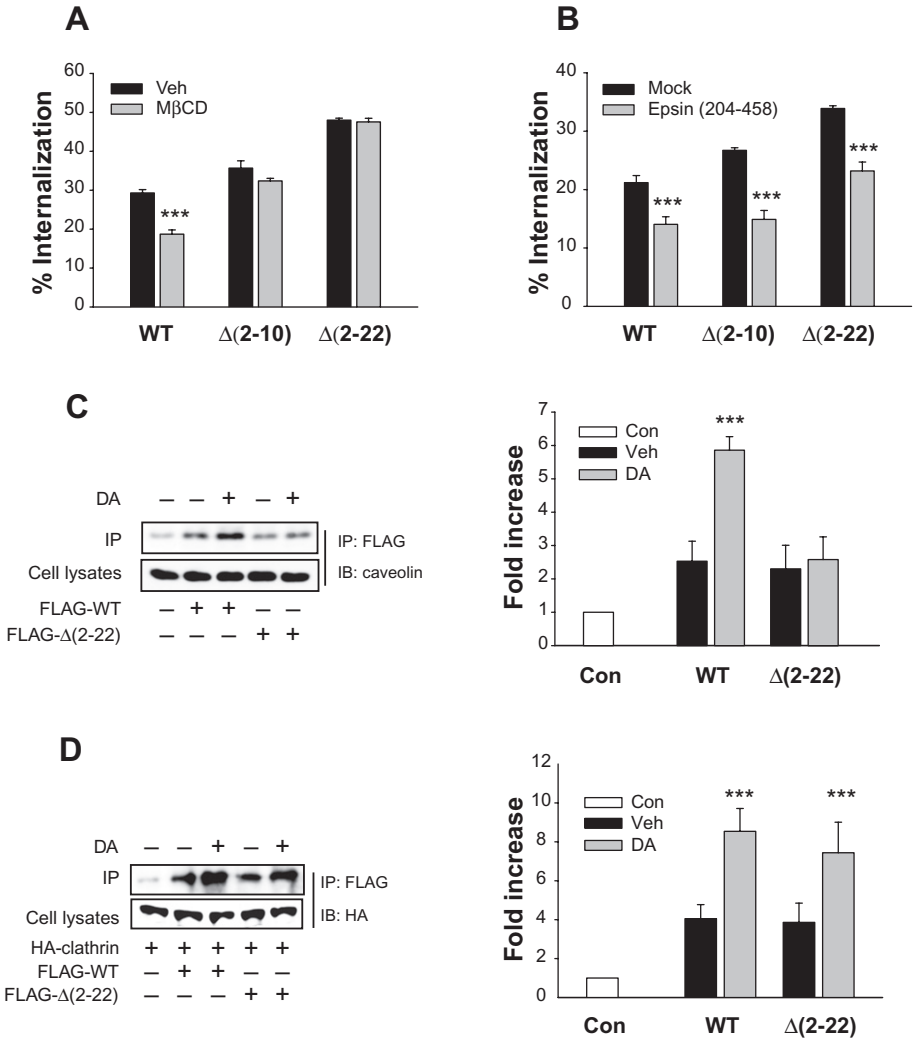

Figure 5

Effects of shortening of the N-terminal region on the endocytic properties of the dopamine D_2 receptor (D_2R). (A) Effects of MβCD treatment on agonist-induced internalization of WT-D_2R and N-terminal deletion mutants. Cells expressing each D_2R construct were treated with 3 mM MβCD for 30 min, then treated with 10 μM of dopamine for 1 h. ***$P < 0.001$ compared with vehicle-treated group. Results are representative of three independent experiments. (B) Effects of epsin(204–458), a dominant negative mutant of epsin, on the internalization of WT-D_2R and N-terminal deletion mutants. Cells transfected with each corresponding combination of DNA constructs were treated with 10 μM dopamine for 1 h. ***$P < 0.001$ compared with mock-transfection group. Results are representative of three independent experiments. (C) Interaction bewteen caveolin-1 and WT-D_2R or Δ(2–22)-D_2R. Cells expressing FLAG-tagged WT-D_2R or Δ(2–22)-D_2R were treated either with vehicle or with 10 μM dopamine for 30 min. Cell lysates were immunoprecipitated with FLAG beads, analysed by SDS-PAGE and immunoblotted with antibodies to caveolin-1. The data represent results from three independent experiments with similar outcomes. ***$P < 0.001$ compared with the vehicle group. (D) Interaction between clathrin and WT-D_2R or Δ(2–22)-D_2R. Cells expressing haemagglutinin (HA)-tagged clathrin and FLAG-tagged WT-D_2R or Δ(2–22)-D_2R were treated either with vehicle or with 10 μM dopamine for 30 min. Cell lysates were immunoprecipitated with FLAG beads, analysed by SDS-PAGE and immunoblotted with antibodies to HA. The data represent results from three independent experiments with similar outcomes. ***$P < 0.001$ compared with vehicle group.

To answer this question, eight amino acids coding for the FLAG epitope (DYKDDDDK) were added to the N-terminal region of Δ(2–10)- and Δ(2–22)-D_2 receptors. If the length of the N-terminal region were the determining factor, it was expected that the properties of Δ(2–10)- and Δ(2–22)-D_2 receptors would change to those of the WT-D_2 receptor and Δ(2–10)-D_2 receptor, respectively. As shown in Figure 6A, the total expression of D_2 receptors ([^{3}H]-spiperone binding) was restored to normal levels but the surface expression ([^{3}H]-sulpiride binding) was either decreased [FLAG-Δ(2–10)] or was the same [FLAG-Δ(2–22)] compared with Δ(2–10)- and Δ(2–22)-D_2 receptors (see Figure 1B and C), suggesting that the specific sequence rather than the relative length of the N-terminal region is the critical factor that determines the correct expression of the D_2 receptor on the cell surface.

Attachment of the FLAG epitope at the N-terminal region of the Δ(2–22)-D_2 receptor resulted in marginally improved

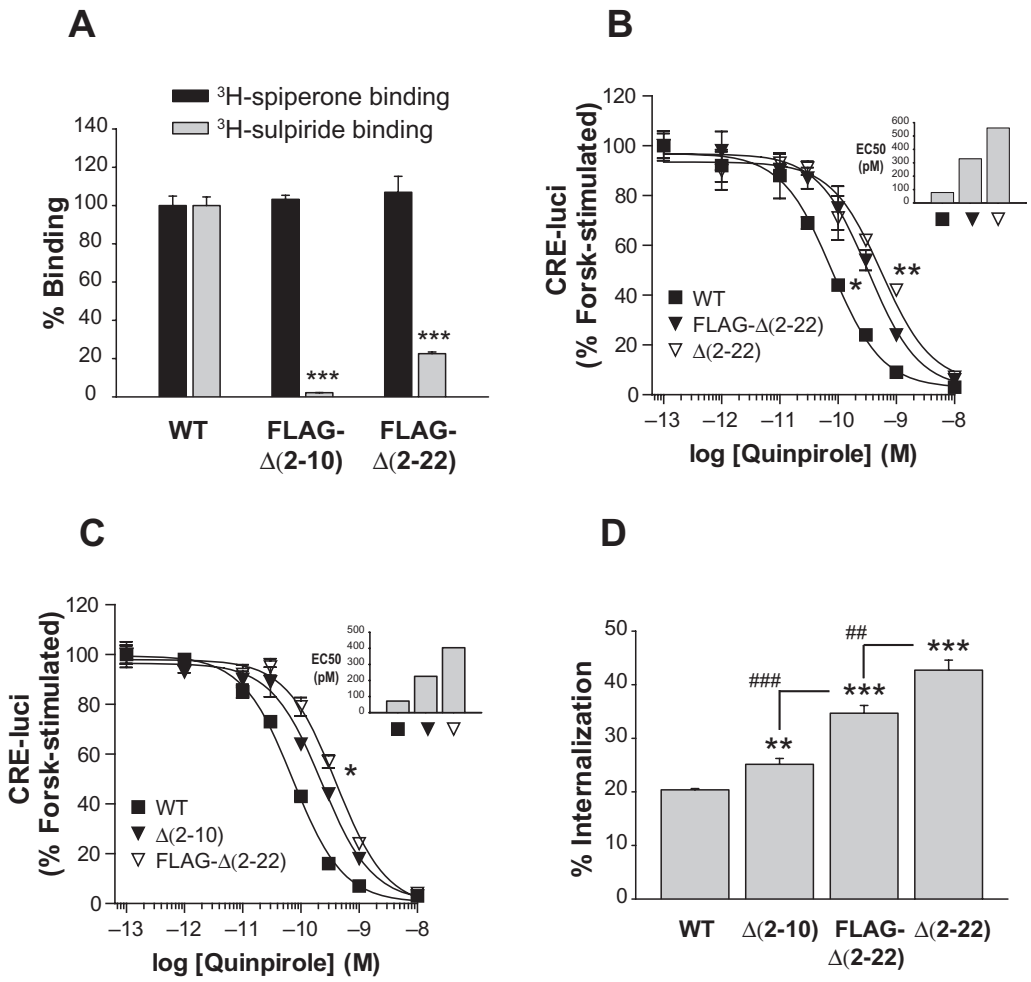

Figure 6

Effects of adding a foreign extension on cell surface binding, signalling and internalization of N-terminal deletion mutants of dopamine D_2 receptor (D_2R). (A) Effects of attachment of the non-specific M2 FLAG sequence (DYKDDDDK) to the N-terminal deletion mutants of D_2R on the cell surface expression. Cells were transfected with 2 μg of each construct and the receptor expression was determined through binding studies with [^{3}H]-spiperone and [^{3}H]-sulpiride. The binding ratio of FLAG-Δ(2–10) and FLAG-Δ(2–22) were normalized to that of WT. ***$P < 0.001$ compared with WT. (B) Effects of attachment of M2 FLAG sequence on the signalling of Δ(2–22)-D_2R. **$P < 0.01$ when Δ(2–22) group was compared with WT group. *$P < 0.05$ when FLAG-Δ(2–22) group was compared with WT group. (C) Comparison of signalling properties between Δ(2–10)-D_2R and FLAG-Δ(2–22)-D_2R. *$P < 0.05$ when FLAG-Δ(2–22) group was compared with WT group. (D) Effects of attachment of M2 FLAG sequence on the internalization of Δ(2–22)-D_2R. **, ***$P < 0.01$, 0.001 compared with WT group, respectively; $^{\#\#}P < 0.01$ when FLAG-Δ(2–22) group was compared with Δ(2–22) group; $^{\#\#\#}P < 0.001$ when FLAG-Δ(2–22) group was compared with Δ(2–10) group. Results are representative of three independent experiments.

signalling (Figure 6B), but failed to reach the level of Δ(2–10)-D_2 receptor, which has an N-terminal region of similar length (Figure 6C). Attachment of the FLAG epitope at the N-terminal region of the Δ(2–22)-D_2 receptor also significantly decreased internalization compared with the Δ(2–22)-D_2 receptor, but it did not reach the level of the Δ(2–10)-D_2 receptor (Figure 6D). The surface expression of FLAG-Δ(2–10)-D_2 receptors was too low to conduct reliable studies (Figure 6A). These results suggest that the surface expression of D_2 receptors is critically regulated by the specific amino acid sequence within the N-terminal region. In the case of signalling and internalization of D_2 receptors, both the specific sequence and the relative length of the N-terminal region play specific roles.

The specific amino acid sequence of the N-terminal region does not affect the ligand-binding or signalling properties

By shortening the N-terminal region or by replacing part of it with a non-specific sequence (FLAG), we showed that the N-terminal region of D_2 receptors controls various receptor functions and characteristics. However, the relative importance of the length or specific amino acid sequence of the N-terminal region for various receptor functions cannot be clearly resolved because the appropriateness of the FLAG sequence as a substitute for the endogenous N-terminal region of D_2 receptor cannot be guaranteed. The foreignness of the FLAG sequence might have altered the receptor func-

tions regardless of the relative length of N-terminal region. Therefore, to reach a clearer conclusion, it would be reasonable to substitute the N-terminal region of D_2 receptor with that of another GPCR, which has a high degree of overall sequence homology but distinct functional features.

D_2 and D_3 receptor, which have a high degree of overall sequence homology, share most signal transduction pathways (Gurevich and Joyce, 1999; Diaz *et al.*, 2000; Joseph *et al.*, 2002), but the D_2 receptor usually signals more efficiently than the D_3 receptor (Robinson and Caron, 1996). In addition, they possess distinct intracellular trafficking properties; that is, the D_2 receptor undergoes mainly agonist-induced receptor internalization (Kim *et al.*, 2001), but the D_3 receptor undergoes mainly PKC-dependent internalization (Cho *et al.*, 2007). The N-terminal regions of D_2 and D_3 receptors show low sequence homology and the D_2 receptor contains five more amino acid residues than the D_3 receptor. If the N-terminal region controls the intracellular trafficking and signalling of GPCRs in a sequence-specific manner as reported for the IL-8 receptor subtypes CXCR1 and CXCR2 (Prado *et al.*, 2007), the switching of the N-terminal regions should reverse the properties of the D_2 and D_3 receptors in terms of intracellular trafficking and signalling properties.

When these chimeric receptors were characterized, the ligand-binding properties of D_2 and D_3 receptors were not altered by the switching of their N-terminal regions (Supporting Information Figure S3A and B), again confirming that the N-terminal region of the catecholamine subgroup of the rhodopsin family of GPCRs is not involved in ligand binding. Furthermore, if the specific amino acid composition of the N-terminal region determines the signalling efficiency of D_2 and D_3 receptors, the signalling efficiency of D_2 and D_3 receptors should be altered when their N-terminal regions are switched. However, attachment of the N-terminal region of the D_3 receptor to the D_2 receptor (D_2 receptor-D_3NT) (Supporting Information Figure S3C) or attachment of the N-terminal region of the D_2 receptor to the D_3 receptor (D_3 receptor-D_2NT) (Supporting Information Figure S3D) did not alter their signalling efficiency when their surface expressions were adjusted to similar levels. These results suggest that neither the specific sequence nor the length of the N-terminal region is a critical factor that controls the signalling of D_2 and D_3 receptors.

The specific amino acid sequence of the N-terminal region determines the subcellular localization of D_2 and D_3 receptors

As described previously, D_2 and D_3 receptors mainly undergo agonist-induced and PKC-mediated internalization, respectively. If the specific amino acid sequence within the N-terminal region determines the internalization properties of D_2 or D_3 receptors, a chimeric D_2 receptor whose N-terminal region is switched with that of the D_3 receptor (D_2 receptor-D_3NT) should show decreased agonist-induced internalization but increased PKC-mediated internalization. Meanwhile, D_3 receptor-D_2NT should exhibit decreased PKC-mediated internalization. As shown in Figure 7A, switching the N-terminal regions did not noticeably change either dopamine-induced or PKC-mediated internalization of WT-D_2 receptors or WT-D_3 receptors. These results suggest that the specific sequence within the N-terminal region is not a critical factor for determining the internalization properties of D_2 receptors. In contrast to the results from the exchange of the N-terminal regions, exchange of the second and third intracellular loops completely changed the internalization properties (Figure 7B), indicating that the amino acid sequence of the second and third intracellular loops is the critical factor that determines the internalization of D_2 and D_3 receptors.

One of the characteristic differences between D_2 and D_3 receptors is their relative subcellular localization. Specifically, a larger fraction of D_3 receptors is located intracellularly compared with D_2 receptors (Kim *et al.*, 2001). To determine the roles of the N-terminal region in the subcellular localization of D_2 and D_3 receptors, the ratio of intracellular binding/total binding was compared. As reported previously (Kim *et al.*, 2001), the ratios of intracellularly located D_2 and D_3 receptors expressed in HEK-293 cells were around 17 and 28%, respectively. To determine the roles of the N-terminal regions on the subcellular localization of D_2 and D_3 receptors, the values of [^{3}H]-sulpiride binding (cell surface binding) were normalized to [^{3}H]-spiperone binding (total binding). As shown in Figure 7C, the relative ratio of cell surface binding/total binding of D_2 receptors (normalized to WT-D_2 receptors) decreased as the N-terminal region was replaced with that of D_3 receptors. In contrast, the ratio of cell surface binding/total binding of D_3 receptors increased as the N-terminal region was replaced with that of D_2 receptors (normalized to WT-D_3 receptors). These results were confirmed by confocal microscopy. As shown in Figure 7D, D_2 receptor-(D_3NT) was more abundantly localized intracellularly than the WT-D_2 receptor. On the other hand, the WT-D_3 receptor was more abundantly expressed intracellularly than the D_3 receptor-(D_2NT) (compare D_3 receptor with D_3 receptor-D_2NT). As reported previously (Kim *et al.*, 2008), the subcellular distribution of FLAG-D_3 receptors was similar to that of WT-D_3 receptors (data not shown). The relative length of the N-terminal region does not seem to be the determining factor in the subcellular localization because the FLAG-D_3 receptor, which has nine more amino acid residues within the N-terminal region than the WT-D_3 receptor, still showed virtually the same level of surface expression as WT-D_3 receptors (Figure 7C).

Glycosylation sites in the N-terminal region are involved in the regulation of subcellular localization and trafficking of D_2 receptors

As shown in Figure 1A, the NT of the D_2 receptor contains three potential N-linked glycosylation sites (N^5, N^{17}, N^{23}) and the NT of the D_3 receptor contains two potential N-linked glycosylation sites (N^{12}, N^{19}). Roles of glycosylation in the NT of GPCRs have been reported for their correct expression on the cell surface (He *et al.*, 2002; Xiao *et al.*, 2011); hence, acceleration of receptor internalization when the potential glycosylation sites were mutated (Tansky *et al.*, 2007). As the N-terminal deletion mutants of D_2 receptors are missing one or two potential glycosylation sites, the involvement of glycosylation in the cell surface expression, internalization and signalling was studied. For this, three potential N-linked glycosylation sites (5th, 17th, 23rd asparagine residues) located within the NT of the D_2 receptor were mutated (D_2 receptor-GlyX).

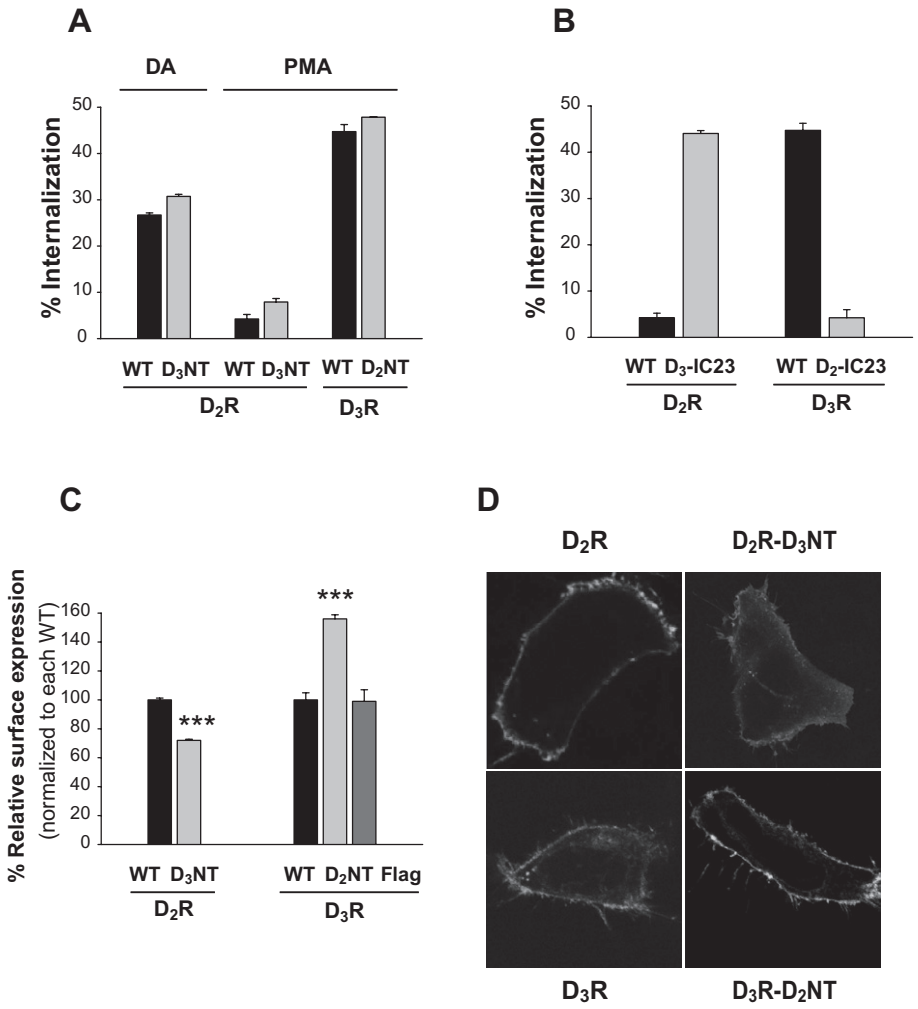

Figure 7

Effects of switching N-terminal regions on the internalization and subcellular localization of the D_2 receptor (D_2R) and D_3R. (A) Effects of switching N-terminal regions on the internalization of D_2R and D_3R. Cells expressing each construct were treated with 10 μM dopamine or 1 μM PMA for 1 h. (B) Effects of switching intracellular loops on the internalization of D_2R and D_3R. Cells expressing each construct were treated with 1 μM PMA for 1 h. D_2R-(D_3-IC23) and D_3R-(D_2-IC23) represent D_2R and D_3R whose second and third intracellular loops are switched with those of D_3R and D_2R, respectively. (C) Effects of switching N-terminal regions on the subcellular localization of D_2R and D_3R. Cells expressing each construct were incubated with 2 nM [^{3}H]-spiperone or 2.2 or 7.2 nM [^{3}H]-sulpiride in the presence or absence of 10 μM haloperidol. [^{3}H]-sulpiride binding/[^{3}H]-spiperone binding that represents [cell surface binding]/[total binding] was normalized for WT-D_2R and changes in their ratio were shown for WT-D_3R and each N-terminal region mutant. ***$P < 0.001$ compared with each WT group. (D) Comparison of confocal images of WT and N-terminal-switched D_2R and D_3R. Cells were transfected with GFP-tagged WT-D_2R, D_2R-D_3NT, WT-D_3R and D_3R-D_2NT. After 24 h, cells were transferred to confocal dishes. The next day, the cells were observed with a laser scanning confocal microscope (TCS SP5/AOBS/Tandem, Leica). Results are representative of two independent experiments.

As shown in Figure 8A, mutation of potential glycosylation sites caused a decrease in the surface expression of D_2 receptors as with the N-terminal deletion mutants (Figure 1B and C). These results were also confirmed by the subcellular localization of GFP-tagged receptors (Figure 8B). The WT-D_2 receptor was mainly observed on the plasma membrane but a significant fraction of the D_2 receptor-GlyX was found in the cytosolic region. The internalization of the D_2 receptor-GlyX was higher than that of the WT-D_2 receptor (Figure 8C); however, the signalling of the D_2 receptor was not significantly altered by mutation of the glycosylation sites (Figure 8D). These results suggest that the major roles of glycosylation of the D_2 receptor in the N-terminal region are the regulation of correct surface expression.

Results in Figures 6 and 7 show that the NT of the D_2 receptor can be substituted with that of the D_3 receptor with no noticeable changes in the subcellular localization and internalization of the D_2 receptor. On the other hand, the hybrid D_2 receptor in which the FLAG epitope was added to the truncated NT (FLAG-Δ(2–22)-D_2 receptor or FLAG-Δ(2–10)-D_2 receptor) showed completely different properties from those of WT-D_2 receptor. Since D_2 receptor-GlyX behaved similarly to the hybrid D_2 receptors, we tested whether the glycosylation sites are the determining factor. For this, we

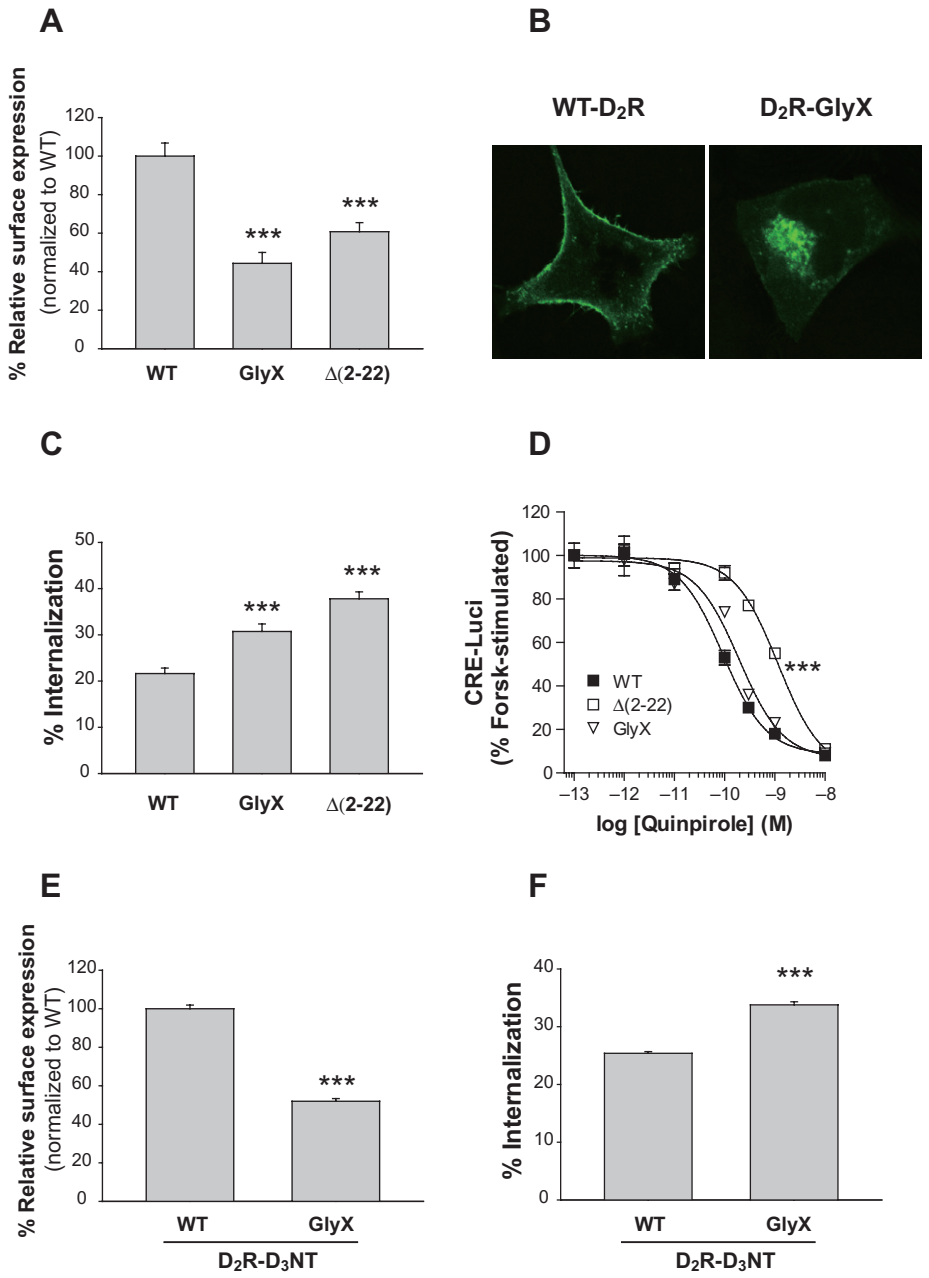

Figure 8

Effects of mutations on potential N-terminal glycosylation sites on the cell surface binding, signalling and internalization of dopamine D_2 receptor (D_2R). (A) Effects of deletion of the N-terminal tail or mutations of potential glycosylation sites on the cell surface expression. Three potential N-linked glycosylation sites on the N-terminal tail (N^5, N^{17}, N^{23}) were mutated to glutamine (Q) residues (GlyX). Cells were transfected with DNA constructs of WT-D_2R, D_2R-GlyX and Δ(2–22)-D_2R at the ratio of 1:10:7. Receptor expression was determined through binding studies with [^{3}H]-spiperone and [^{3}H]-sulpiride. The binding ratio of D_2R-GlyX and Δ(2–22)-D_2R was normalized to that of WT-D_2R. ***$P < 0.001$ compared with WT. (B) Comparison of confocal images of WT-D_2R and D_2R-GlyX. Cells were transfected with GFP-tagged WT-D_2R or D_2R-GlyX. Images were captured as in Figure 7B. (C) Comparison of the internalization properties of WT-D_2R with those of D_2R-GlyX. Cells were transfected with the DNA constructs as in (A), and receptor internalization was determined as described in the Methods section. ***$P < 0.001$ compared with WT group. (D) Comparison of signalling properties between WT-D_2R and D_2R-GlyX. Cells were transfected with the DNA constructs as in (A) and the receptor signalling was determined by the inhibition of cAMP production as described in the Methods section. ***$P < 0.001$ compared with WT group. (E) Comparison of relative surface expression between D_2R-D_3NT and GlyX mutant of D_2R-D_3NT in which two potential glycosylation sites were mutated (D_2R-D_3NT-GlyX). Receptor expression was determined through binding studies with [^{3}H]-spiperone and [^{3}H]-sulpiride. The binding ratio of D_2R-D_3NT and D_2R-D_3NT-GlyX was normalized to that of D_2R-D_3NT. ***$P < 0.001$ compared with D_2R-D_3NT group. (F) Comparison of internalization properties between D_2R-D_3NT and GlyX mutant of D_2R-D_3NT. Cells were transfected with the DNA constructs of D_2R-D_3NT and D_2R-D_3NT-GlyX at the ratio of 1:7, and the receptor internalization was determined as described in the Methods section. ***$P < 0.001$ compared with D_2R-D_3NT group. Results are representative of three independent experiments.

additionally created a GlyX mutant in the NT of D_2 receptor-D_3NT in which two potential glycosylation sites (N^{12}, N^{19}) were mutated to glutamine residues. As shown Figure 8E and F, the cell surface expression of this mutant was decreased and its internalization increased compared with D_2 receptor-D_3NT in which two potential glycosylation sites were intact. These results suggest that glycosylation in the NT of the D_2 receptor could be the critical factor that determines the stable expression of D_2 receptors on the plasma membrane.

Discussion

The roles of the N-terminal regions of GPCRs in receptor functions, such as ligand binding, surface expression, signalling and internalization, vary depending on the size and structure of the N-terminal extension. It is well-established that the N-terminal regions of certain GPCRs are somehow involved in ligand binding (Bockaert and Pin, 1999). These GPCRs include secretin-like (family 2), metabotropic glutamate-like (family 3) and some of the rhodopsin-like (family 1) GPCRs that bind to peptides. Other than this, however, the roles of the N-terminal region remain largely unknown. It has even been reported that the roles of the N-terminal region vary within the same family of GPCRs. For example, deletion of the N-terminal regions of different neuropeptide Y receptor subtypes results in different properties in terms of signal transduction, ligand binding and cell surface expression (Lindner *et al.*, 2009).

The N-terminal regions of the rhodopsin-like GPCR family have been the least studied among families of GPCRs, and their roles in signalling and internalization have not been reported. In this study, we wanted to understand the functional roles of the N-terminal region of the rhodopsin-like GPCR family by using the D_2 receptor as an example. Two different strategies were employed to get answers to these questions; either the N-terminal region was shortened or was replaced with other sequences such as a non-specific FLAG sequence or the N-terminal region of the D_3 receptor. The shortening of the N-terminal region prevented the correct expression of D_2 receptors on the cell surface, increased the receptor internalization (Figures 1 and 3) and altered the endocytic routes (Figure 5). Replacement of part or the whole of the N-terminal region of the D_2 receptor with other sequences prevented the characteristic expression of receptor proteins on the cell surface (Figures 6A and 7C). Therefore, results from different set of experiments indicate that the N-terminal region of the D_2 receptor is involved in maintaining the receptor proteins in a stable conformation on the cell surface.

In some GPCRs such as the cannabinoid receptor 1, α1-adrenoceptor and GPR37 (Andersson *et al.*, 2003; Hague *et al.*, 2004; Dunham *et al.*, 2009), it was reported that the truncation of the NT increases their expression on the plasma membrane. Interestingly, these receptors commonly possess a relatively long N-terminal and show low expression levels on the plasma membrane. On the other hand, the D_2 receptor contains a short NT and shows good expression on the plasma membrane (Figure 1D). These results suggest that the N-terminal regions of GPCRs control the surface expression of receptor proteins but their effects vary depending on the structural features of each receptor. The molecular mechanism by which the NT controls the endocytic process is unclear, but it can be speculated on based on the structural orientation of receptor proteins in the process of endocytosis. Since the N-terminal sequences are on the inside of endocytic vesicles, it is possible that such sequences might react to low pH inside these vesicles and by some allosteric mechanism, transmit information to regions of the receptor on the outside, where they interact with trafficking proteins.

It is noticeable that the endocytosis of D_2 receptors in the caveolae but not in clathrin-coated pits is selectively inhibited after the N-terminal had been shortened (Figure 5). Clathrin-coated pits and caveolae are two major microdomains of the plasma membrane where endocytosis of GPCRs occurs. The endocytosis of GPCRs through clathrin-coated pits is relatively well characterized. Receptors that are phosphorylated by GRKs bind to β-arrestins and the receptor/β-arrestin complex combines with other proteins such as AP-2 to form vesicles coated with clathrin. On the other hand, the mechanism of the endocytic process through caveolae remains unclear. More fundamental studies are needed to understand the functional meaning of the selective blockade of D_2 receptor endocytosis in the caveolae with deletion of the N-terminal region.

The finding that endocytosis of the D_2 receptor was increased with the deletion of the N-terminal region in which the endocytosis in the caveolae is abolished was unexpected. It would be interesting to know what kind of endocytic pathway other than clathrin-coated pits or caveolae is involved in the rapid internalization of N-terminal deletion mutants. At this point, it could be speculated that correct integration of receptor proteins into the plasma membrane, which requires balanced charge distribution, is impaired in N-terminal deletion mutants, resulting in impaired surface expression (Figure 1C). Receptors in an unstable conformation in the plasma membrane might easily be mobilized into the cytosolic region in response to agonist stimulation. These results again suggest that the N-terminal region of the D_2 receptor is involved in maintaining the receptor proteins in a stable conformation so that the endocytic process of the D_2 receptor is more strictly regulated. This would explain how the signalling of D_2 receptors was progressively inhibited as the N-terminal region was shortened, which is presumably caused by the marked increase in receptor internalization. Therefore, it is concluded that the series of cellular events evoked by shortening of the N-terminal tail was caused by a change in receptor conformation that caused the receptor proteins to be placed on the plasma membrane in a more unstable state. It was reported that caveolae targeting the Epstein-Barr virus latent membrane protein 1 is mediated through its N-terminal region (Rothenberger *et al.*, 2002). Our results also show that the NT of GPCRs are involved in their differential localization in the plasma membrane microdomains and provide some guidance to other researchers so that the exact molecular mechanisms can be elucidated in the future.

Previous studies have shown that the N-terminal region plays an important role in the internalization of GPCRs, but the results are contradictory. In the case of the neuropeptide Y receptor, adding non-specific amino acid residues to the first TM of the N-terminally truncated receptor was sufficient

to recover receptor activity (Lindner *et al.*, 2009). On the other hand, the IL receptors CXR1 and CXR2 needed specific amino acid sequences in the N-terminal region for proper signalling and internalization (Prado *et al.*, 2007). More extensive studies on a variety of GPCRs are needed to establish the key criteria that decide whether a simple physical extension in the N-terminal region is needed or whether a combination of specific amino acid residues is required for them to function properly.

Our results in Figure 6 show that adding a FLAG epitope to the N-terminal region of the deletion mutants did not provide decisive answers for this question. The specific sequence within the N-terminal region is definitely needed for correct positioning of receptor proteins on the cell surface. However, both factors seem to be involved in the signalling and internalization of the D_2 receptors. A clearer conclusion could be reached if more functional data were provided by increasing the sequence variety of the added foreign extension. However, this approach has an intrinsic limitation in that it is difficult to reach a clear conclusion unless it can be proved that the changes in receptor functions are not caused by the foreignness of the added non-specific amino acid residues. Hence, exchange of the N-terminal regions between D_2 and D_3 receptors could provide a more decisive answer to resolve whether the length or the specific sequence of the N-terminal tail determines receptor function. Studies with the chimeric D_2 and D_3 receptors (Figures S3 and 7) confirmed the previously established notion that the intracellular loops are the major regions that determine signalling and intracellular trafficking. However, it is notable that the surface expression of receptor proteins was largely determined by the N-terminal regions and this confirms the conclusion obtained from the FLAG epitope extension studies (Figure 6). Figure 8A–D show that glycosylation in the NT could be a critical factor that determines the stable expression of receptor proteins on the cell surface. These results might explain why the NT of the D_3 receptor, which contains glycosylation sites but not FLAG epitope, could substitute for the NT of the D_2 receptor without inducing significant changes in receptor functions or properties (Figures 6 and 7). It is noticeable that the enhancement of internalization was more prominent with the Δ(2–22)-D_2 receptor than the D_2 receptor-GlyX, suggesting that glycosylation sites as well as other regions in the NT are involved in the regulation of receptor internalization.

In conclusion, the N-terminal region of the D_2 receptor does not participate in a specific ligand-binding pocket but is likely to be important for receptor trafficking, since the gradual shortening of the N-terminal region increased the internalization rate of the D_2 receptor. The most important role of the N terminus seems to be to ensure the correct receptor structure and integration into the cell membrane, which is largely determined by the glycosylation in the NT.

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Roles of GRK2 and β-arrestins in the enhancement of D_2R internalization accompanied by shortening of the N-terminal region. (A) Knockdown of endogenous GRK2. Cells were transfected with scrambled shRNA (Con) or GRK2 shRNA in pcDNA3.0 by a calcium phosphate method and maintained in the culture media that contained 500 $\mu g \cdot ml^{-1}$ of G418. Selected clones were tested for the expression of endogenous GRK2 and actin by immunoblotting the cell lysates with antibodies to GRK2 and actin. The expression GRK2 was inhibited by about 75%. (B) Effects of knockdown of endogenous GRK2 on agonist-induced internalization of WT-D_2R and N-terminal deletion mutants. Cells expressing each D_2R construct were treated with 10 μM of DA for 1 h, washed with serum-free media and then incubated with 2.2 nM of [^{3}H]-sulpiride for 150 min at 4°C. Receptor expression levels were maintained around 0.5 $pmol \cdot mg^{-1}$ protein. *, **$P < 0.05$, 0.01 compared with each Con-KD group respectively. (C) Knockdown of endogenous β-arrestins. Cells were stably transfected with scrambled shRNA (Con) or β-arrestin2 shRNA in pcDNA3.0. Cells that stably express β-arrestin2 shRNA were transfected with β-arrestin1 shRNA in pcDNA3.1/Zeo(+). Cells were maintained in media that con-

tained 500 μg·mL^{-1} of G418 and 250 μg·mL^{-1} of zeocin. HEK-293 cells stably expressing shRNA constructs of scrambled sequence or β-arrestin1/β-arrestin2 were selected. Levels of endogenous β-arrestins were determined by immunoblotting. Cell lysates were blotted with antibodies to β-arrestin1, β-arrestin2 and actin. The expression of β-arrestin1 and β-arrestin2 were inhibited by 70 and 85% respectively. (D) Effects of knockdown of endogenous β-arrestins on agonist-induced internalization of WT-D_2R and N-terminal deletion mutants. Cells expressing each D_2R construct were treated with 10 μM of DA for 1 h. Receptor expression levels were maintained around 0.5 pmol·mg^{-1} protein. *, **, ***$P < 0.05$, 0.01, 0.001, respectively, compared with each Con-KD group. (E) Comparison of the interaction between GRK2 and WT-D_2R or Δ(2-22)-D_2R. Cell lysates were prepared from each experimental group and immunoprecipitated with FLAG beads, and the immunoprecipitates were analysed with SDS-PAGE and immunoblotted with antibodies to GRK2. Receptor expression levels were maintained around 0.5 pmol·mg^{-1} protein. (F) Comparison of the interaction between β-arrestin2 and WT-D_2R or Δ(2-22)-D_2R. Experiments were conducted as in Supporting Information Figure S1E except that GRK2 was replaced with β-arrestin2.

Figure S2 Comparison between WT-D_2R and Δ(2-22)-D2R for the colocalization with endogenous caveolin-1. Immunocytochemical studies were conducted as described in the Methods section. Cells were labelled with antibodies to caveolin-1 at a 1:1000 dilution and with Alexa 594-conjugated anti-mouse antibodies at a 1:250 dilution. (A, B) Cells were transfected with GFP-tagged WT-D_2R and treated with vehicle (A) or 10 μM of DA (B) for 30 min. The arrows show the co-localization of D_2R and caveolin-1 at the plasma membrane (A) and in the same endocytic vesicles (B). (C, D) Cells were transfected with GFP-tagged Δ(2-22)-D_2R and treated with vehicle (A) or 10 μM of DA (B) for 30 min.

Figure S3 Effects of switching N-terminal regions between D_2R and D_3R on the ligand-binding properties of D_2R and D_3R. (A) Effects of replacement of the N-terminal region with that of D_3R on the ligand-binding properties of D_2R. Cells expressing D_2R or D_2R-D_3NT were incubated with 2.2 nM of [^{3}H]-sulpiride and increasing concentrations of DA in the absence and presence of 10 μM of haloperidol for 1 h at 37°C. Cells were washed with ice-cold, serum-free media three times. Cells were lysed with 1% SDS and the remaining radioactivity was measured by liquid scintillation counting. (B) Effects of replacement of the N-terminal region with that of D_2R on the ligand-binding properties of D_3R. Cells expressing D_3R or D_3R-D_2NT were incubated with 7.2 nM of [^{3}H]-sulpiride and increasing concentrations of quinpirole in the absence and presence of 10 μM of haloperidol for 1 h at 37°C. (C) Effects of replacement of the N-terminal region with that of D_3R on the signalling properties of D_2R. Reporter gene assays were conducted in cells expressing D_2R or D_2R-D_3NT. Receptor expression levels were maintained around 1.2 pmol·mg^{-1} protein. Effects of switching N-terminal regions on the signalling properties of D_2R and D_3R. Reporter gene assays were conducted from cells expressing D_2R, D_3R, or D_3R-D_2NT. Receptor expression levels were maintained around 0.9 pmol·mg^{-1} protein.

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