SH3 Binding Domains in the Dopamine D4 Receptor[†]

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ABSTRACT: The dopamine D4 receptor is a G protein-coupled receptor (GPCR) that belongs to the dopamine D2-like receptor family. Functionally, the D2-like receptors are characterized by their ability to inhibit adenylyl cyclase. The dopamine D4 receptor as well as many other catecholaminergic receptors contain several putative SH3 binding domains. Most of these sites in the D4 receptor are located in a polymorphic repeat sequence and flanking sequences in the third intracellular loop. Here we demonstrate that this region of the D4 receptor can interact with a large variety of SH3 domains of different origin. The strongest interactions were seen with the SH2−SH3 adapter proteins Grb2 and Nck. The repeat sequence itself is not essential in this interaction. The data presented indicate that the different SH3 domains in the adapter proteins interact in a cooperative fashion with two distinct sites immediately upstream and downstream from the repeat sequence. Removal of all the putative SH3 binding domains in the third intracellular loop of the dopamine D4 receptor resulted in a receptor that could still bind spiperone and dopamine. Dopamine could not modulate the coupling of these mutant receptors to adenylyl cyclase and MAPK, although dopamine modulated receptor−G protein interaction appeared normal. The receptor deletion mutants show strong constitutive internalization that may account for the deficiency in functional activation of second messengers. The data indicates that the D4 receptor contains SH3 binding sites and that these sites fall within a region involved in the control of receptor internalization.

The dopamine receptors belong to the large family of seven transmembrane G protein-coupled receptors (GPCR).[1] To date, five different dopamine receptor genes have been identified and cloned from mammalian species (see ref *1* for review). The dopamine receptors are classified into two subgroups according to their structural, pharmacological, and functional characteristics: the dopamine D1-like receptors (D1 and D5), which can activate adenylyl cyclase, and the D2-like receptors (D2, D3, and D4), which can inhibit adenylyl cyclase upon agonist binding.

The physiological role of the dopamine D4 receptor is still unclear. This receptor appears to be predominantly expressed in the mesocortical and mesolimbic areas of the brain, most notably the GABA-ergic interneurons of the frontal cortex (*2*, *3*). In addition, D4 receptor mRNA has been detected in the retina and the heart (*4*, *5*). The distribution profile of this receptor and its relatively high affinity for clozapine, as compared to the D2 receptor, has resulted in the implication of this site as an important target for atypical neuroleptics (*2*, *6*). Genetic association and linkage studies suggest a role of the D4 receptor in attention deficit hyperactivity disorder, novelty seeking, and Tourette's syndrome, although the latter two findings have been disputed (*7*−*12*). Similar studies have not supported a role for this receptor in schizophrenia and bi-polar disorder (*13*, *14*).

The human dopamine D4 receptor has a variable number of tandem repeats (VNTR) within the third intracellular loop domain that is not observed in other dopamine receptors (*15*). This polymorphic repeat consists of between 2 and 10 repeated units of 16 amino acids in length. To date, 20 different receptor protein variants have been found for this polymorphism (*15*−*17*). The first (α) and last (ζ) repeat units, however, are invariant, irrespective of the total number of repeat units (*15*−*17*). D4 receptor genes encoding receptors with two (D4.2), four (D4.4), or seven (D4.7) repeat units are the most abundant in the human population. A similar VNTR is also present in various nonhuman primate species but is not seen in rodents (*15*, *18*). With respect to functional coupling to adenylyl cyclase, it has been observed that the D4.7 isoform has a 2−3-fold decreased potency for dopamine as compared to the D4.2 and D4.4 isoforms (*19*).

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[1] Abbreviations: GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; VNTR, variable number of tandem repeats; RTK, receptor tyrosine kinase; SH3, Src homology 3; SH2, Src homology 2; GST, glutathione *S*-transferase; HA, hemagglutinin; disintegrations per minute, dpm.

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Examination of the repeat sequence and its immediate surrounding sequences revealed the presence of multiple putative Src homology 3 (SH3) binding motifs in this region. SH3 domains are recognized as modular binding domains for protein−protein interactions that are essential for full functional activity of, for example, the receptor tyrosine kinase (RTK) complex (see ref *20* for review). SH3 domains recognize proline-rich sequence motifs of about 10 amino acids in length that contain a conserved PXXP sequence (*20*−*23*). A scan of the currently identified GPCRs revealed that there were other receptors that also contained potential SH3 binding motifs, including the α- and *β*-adrenergic and muscarinic receptors. To date, the involvement of SH3 domains in seven transmembrane receptor signaling has not been fully investigated (*21, 24*). The identification of such domains could direct the search for additional essential components of the GPCR complex, which may elucidate other functions of the receptors or give further insight into the mechanisms for GPCR signal transduction.

In this study, we demonstrate that the putative SH3 binding domains within the dopamine D4 receptor, the *β*1 adrenergic, and the M4 muscarinic receptor can interact with SH3 domains. In particular, we have investigated the interaction of the D4 receptor with the Src-homology 2 (SH2)−SH3 adapter proteins Nck and Grb2. Furthermore, we show that deletion of all the putative SH3 binding domains in the third intracellular loop of the D4 receptor results in constitutive internalization of the receptor.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Plasmids. D4.4(220−336) encoding most of the third intracellular loop of the human dopamine D4.4 receptor was subcloned into the *Xba*I/*Eco*NI sites of ppoly(A)luc(T7) (Promega) such that most of the luciferase gene from ppoly(A)luc(T7) was removed to provide a start codon and a poly A tail for the in vitro expression of the third intracellular loop of D4.4. The full-length cDNAs of D4.2(1−387), D4.4(1−419), and D4.7-(1−467) (*15*) were subcloned into pBluescript SKII (−) (Stratagene). Human D4.4 deletion mutants (see Figure 1C) D4.(Δ254−315) (*17*), D4.(Δ221−315) (*17*), and D4.(Δ221−337) were created by removing the indicated amino acids from the D4.4(1−419) construct. D4.2(249−280) only codes the first (α) and last repeat (ζ) of the human D4.2 receptor and was subcloned into the *Xba*I/*Eco*RV sites of ppoly(A)-luc(T7). The human dopamine D3 receptor (1−400) (a gift from Dr. J.-C. Schwartz and Dr. P. Sokoloff), the human *β*-adrenergic *β*1 receptor (1−477) (a gift from Dr. M. Caron), and the human M4 muscarinic receptor (1−479) (a gift from Dr. T. Bonner) were subcloned into pBluescript. The region of the rat D4 receptor (224−305) that is homologous to the human D4 receptor repeat and flanking region was subcloned into the *Eco*RV/*Xba*I site of ppoly(A)luc(T7). All constructs made for in vitro expression were placed under the control of the T7 promoter.

A number of glutathione *S*-transferase (GST) fusion protein constructs, encoding either the entire protein or only the SH3 domains, were made. GST-Nck 3SH3, encoding the three SH3 domains (1−255) of the human Nck protein; GST-Crk SH3 (N) encoding the amino terminal SH3 domain of human Crk-II (133−184); and GST-Crk SH3 (C) (238−290) encoding the carboxy terminal SH3 domain of human Crk-II were subcloned into PGEX-2T. GST-Grb2 encoding the full-length human Grb2 protein (1−217), GST-Grb2 SH3 (N) encoding the amino terminal SH3 domain (1−58), and GST-Grb2 SH3 (C) encoding the carboxy terminal SH3 domain (159−217) have been described (*25, 26*). Grb2 (P49L) was created by substituting a conserved proline with a leucine at amino acid residue 49 of the human Grb2 protein. GST-Abl SH3 (65−123 of p210 Bcr-Abl), GST-p85 SH3 encoding the SH3 domain of the bovine p85α protein (1−86), GST-PLC*γ* encoding the human PLC*γ* SH3 domain (792−851), GST-spectrin encoding the chicken α-chain spectrin protein's SH3 domain (967−1025), and GST-c-Src SH3 encoding the chicken c-Src SH3 domain construct have been described (*26*−*28*).

The sequences encoding the third intracellular loop of the human D4.2, D4.4, and D4.7 dopamine receptors (219−304, 219−336, and 219−384, respectively) were subcloned into the *Bam*HI site of the yeast two-hybrid expression vector pACTII. The Crk SH2 domain (10−105 of Crk-II) and the Sos tail were subcloned into pACTII (*29*).

The mouse Abl SH3 domain (65−123 of p210 Bcr-Abl), the entire human Grb2, amino (N) or carboxy (C) terminal domains (1−217, 1−59, and 158−217, respectively), the human PLC*γ* (792−851), the mouse Crk carboxy (C) terminal SH3 domain (235−293), and Nck 3SH3 (1−255) were subcloned into pASI (*30*). The third intracellular loop of the human D4.2(219−304), D4.4(219−336), and D4.7-(219−384) dopamine receptors were subcloned into the *Sma*I/*Sfi*I sites of the yeast two-hybrid expression vector pASI.

D4.7, D4.(Δ221−315), and D4.(Δ221−337) were subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen) and fused either to the HA (hemagglutinin) epitope (YPYDCPDYA) or the signal-Flag epitope sequence at the amino-terminal extracellular domain of the receptors as described previously for the opioid receptors (*31*).

In Vitro Protein−Protein Interaction Assay. Proteins expressing the different GPCRs, or parts thereof, were labeled by T7 polymerase with ^{35}S-L-cysteine [^{35}Cys] (ICN) using a rabbit reticulocyte in vitro transcription/translation system (TNT Coupled Reticulocyte Lysate Systems, Promega) according to the manufacturer's instructions. In vitro product was quantified according to the manufacturer's instructions by liquid scintillation of the purified radiolabeled protein. GST fusion proteins were prepared according to the GST Gene Fusion System protocol (Pharmacia Biotech) either in XL-1 or BL-21 *Escherichia coli* cells. Cells were grown in LB media to an $OD_{600} > 0.6$. Protein synthesis was induced with 1 mM isopropylthiogalactopyranoside for 2 h. The cultures (1.5 mL) were spun down and resuspended in lysis buffer (1× PBS, 1% TritonX-100, 1% Tween 20, 10 mM DTT, 2 *μ*g/mL aprotinin, and 2 *μ*g/mL leupeptin) and sonicated (power 70 for 10 s; Biosonik sonicator, Bronwill Scientific). The lysate was spun down (30 s, 500 g). A sample of 50 *μ*L of a 50% slurry of glutathione beads was added to the lysate and was then incubated with shaking at room temperature for 5 min. The beads were spun down (30 s, 500 g), rinsed three times with fresh lysis buffer (100 *μ*L), and finally resuspended in 300 *μ*L of lysis buffer. The concentration of the purified GST fusion protein was

determined by eluting off the GST fusion protein from the glutathione beads using glutathione (10 mM) and measuring the absorbance at 280 nm. Freshly prepared in vitro transcribed ^{35}Cys-labeled protein (2 μL; 0.5 pmol) was added to the 300 μL glutathione bead preparation and incubated with gentle shaking at 4 °C for 2 h. The beads were spun down, the supernatant was discarded, and the beads were rinsed three times with fresh ice-cold lysis buffer (100 μL). Finally, 30 μL of 1× sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.005% bromophenol blue, 10% glycerol, 2.5% β-mercaptoethanol) was added, heated to 90 °C for 5 min and run on a 8−16% Tris−glycine gel (Novex). The gels were fixed, stained with Brilliant Blue (Sigma), soaked in EN^{3}HANCED (Du Pont), dried on filter paper, and exposed to autoradiography film.

In Vitro Competition and Saturation Analysis. For the competition and saturation assays, the above-described procedure was followed unless otherwise noted. For the competition assay, equivalent amounts of GST-Nck 3SH3 and equivalent amounts of in vitro labeled [^{35}Cys]D4.4(220−336) protein (1 μL; 0.25 pmol), prepared as described above, were co-incubated. A 10-fold higher amount (10 μL; 2.5 pmol) of unlabeled in vitro product D4.4(220−336) or D4.(Δ221−315) was added either 30 min prior, at the same time, or 30 min after the addition of the labeled protein. The interaction was analyzed as described above.

For the saturation assay, equivalent molar amounts of in vitro labeled ^{35}Cys-labeled protein (2 μL; ∼0.5 pmol) was incubated with increasing concentrations of GST, GST-Nck 3SH3, or GST-Grb2 fusion protein to determine the strength of interaction between the various D4 constructs or D4 deletion mutants with Nck 3SH3 or Grb2. The volume of glutathione beads and lysis buffer was kept constant for the various dilutions of GST fusion protein for each saturation curve. After incubation, the beads were washed with 2 vol of 200 μL lysis buffer. Samples were run as described above and exposed to a phosphoimaging cassette overnight. The amount of in vitro product immobilized by GST fusion protein was quantified using the STORM 680 phosphoimager (Molecular Dynamics). The scans were analyzed with ImageQuant for Windows NT software (Molecular Dynamics), and approximations for the kinetic values (K_d and B_{max}) were obtained using Graphpad Prism 2.01 software.

Yeast Two-Hybrid Assays. The yeast two-hybrid assay was performed as previously described (*30*). pASI vectors encoding fusions between the DNA-binding domain of *GAL4* and the different SH3 domains and pACTII vectors encoding the transcriptional activation domain of *GAL4* fused to different fragments described above were transformed into *Saccharomyces cerevisiae* strains Y-153 and Y-187, respectively.

Cell Culture. Clonal selected CHO-K1 cells stably transfected with the HA epitope-tagged D4.7 or the D4 receptor deletion mutants D4.(Δ221−315) and D4.(Δ221−337) in the expression vector pcDNA3 were created and grown as previously described (*19*). Clonal selected HEK-293 cells transfected with HA or Flag epitope-tagged receptors in the pcDNA3 vector were created and grown as previously described (*31*).

Radioligand Receptor Binding. Ligand binding studies were completed as previously described (*17*, *32*). In brief, CHO-K1 cells expressing the D4.7 or the deletion mutant receptors were homogenized (Polytron, Brinkmann Instruments, Westbury, NY; maximum, 15 s) at 4 °C in binding buffer [50 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 5 mM EDTA, and 120 mM NaCl]. The homogenates were centrifuged for 20 min at 39000*g*, and the pellets were resuspended in binding buffer at a concentration of approximately 200 μg/mL. For saturation binding analysis, 250-μL homogenates were incubated in duplicate with increasing concentrations (10−1000 pM) of [^{3}H]-spiperone (98 μCi/mmol). Competition binding analysis was performed by co-incubation of 300 pM [^{3}H]spiperone and increasing concentrations (10^{-11}−10^{-4} M) of dopamine in either the presence or the absence of 200 μM guanilylimido diphosphate (Gpp(NH)p). Nonspecific binding was determined by co-incubation of [^{3}H]spiperone with 1 μM haloperidol. The samples were incubated in a final volume of 1 mL for 2 h at room temperature and then filtered using a cell harvester (Skatron Instruments, Lier, Norway). Radioligand bound to the filters was detected by liquid scintillation counting (Beckman LS6000SC). The density of [^{3}H]ligand binding sites (B_{max}) and dissociation constants of ligands (K_d) were determined by Scatchard analysis. The ligand binding data were analyzed by the nonlinear least squares curve-fitting program Ligand.

GTPγ^{35}S Binding Assay. Membrane preparations were tested for agonist-stimulated GTPγ^{35}S binding. Membranes were incubated (30 °C, 30 min) in duplicate with dopamine (1 nM−100 μM) in a buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM KCl, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 120 mM NaCl, 10 μM GDP, and 0.1−0.2 nM GTPγ^{35}S (1250 Ci/mmol; NEN) in a total volume of 340 μL. Incubations were terminated by rapid filtration onto GF/A filters, and the amount of bound GTPγ^{35}S was determined by scintillation counting. The disintegration per minute (dpm) values were corrected for nonspecific binding, which was determined with 10 μM GTPγS.

cAMP Measurements. cAMP measurements were completed as described previously (*19*, *32*). In brief, cells were plated onto six-well, 35-mm dishes 2 days before the experiment and grown to 100% confluency (∼1.0×10^6 cell/plate). These cells were washed with 2 mL of HBBS buffer (118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 20 mM HEPES, and 0.3 mM isobutyl-1-methylxanthine, pH 7.2). To determine the potency of agonists, D4.7 and D4 deletion mutant expressing cell lines were incubated with 10 μM forskolin in the presence or absence of 10 μM dopamine. The cells were incubated for 30 min at 37 °C in a final volume of 2 mL. At the end of the incubation period, the medium was removed by aspiration, and the cells were harvested in 1 mL of permeabilization buffer [0.05% (vol/vol) Triton X-100 in HBBS buffer]. The samples were vortexed and spun for 5 min at 14 000 rpm in a microcentrifuge. The supernatant was collected, and the cAMP concentration was assayed by RIA (*19*).

MAPK Assay. A MAPK assay, which detected the phosphorylation of Erk1 (p42) and Erk2 (p44) MAP kinases after receptor stimulation, was completed as described by the manufacturer (PhosphoPlus MAPK Antibody Kit, New England Biolabs Inc., MA) with the following changes. Prior to carrying out the drug incubations, the cells were incubated

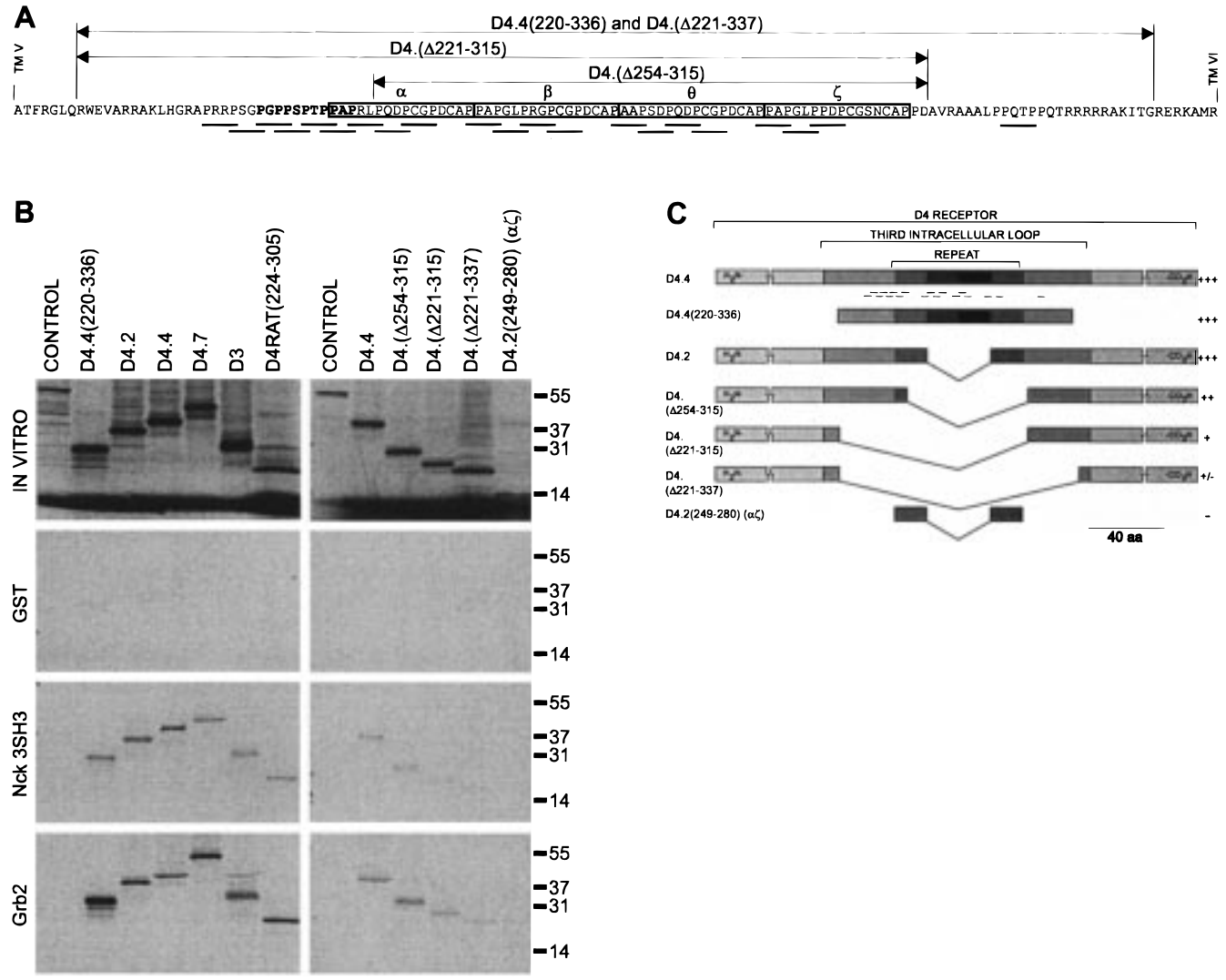


FIGURE 1: Identification of the different dopamine receptors or parts of the human dopamine D4 receptor that interact with the adapter proteins Nck and Grb2. (A) Depicted is the complete amino acid sequence of the third intracellular loop of the human D4.4 receptor. The repeat units ($\alpha\beta\theta\zeta$) are boxed. Horizontal lines highlight the different PXXP motifs. Bold lettering is used to indicate the SH3 binding motifs that meet the more stringent consensus sequence (*22*). The amino acid residues of the third intracellular loop fragment D4.4(220−336) and the amino acid residues removed in the deletion mutants [D4.(△254−315), D4.(△221−315), and D4.(△221−337)] are indicated with arrows. (B) Different in vitro ^{35}Cys-labeled receptor product (0.5 pmol) or control (luciferase) (top panel) was incubated with the GST, GST-Nck 3SH3, or GST-Grb2 fusion proteins (30 μM) (lower panels). The immobilized in vitro product was run on a protein gel and exposed to film (24 h exposure). Molecular masses are indicated in kilodaltons. (C) A schematic of the different parts of the dopamine D4 receptor tested is represented. Interaction is indicated by the following: (+++) very strong, (++) strong, (+) weak, (+/−) very weak.

overnight with serum-free α-MEM. Two hours prior to the experiment, the media was again replaced with fresh serum-free α-MEM. To measure receptor-mediated stimulation of Erk1 and Erk2 phosphorylation, cells were incubated with α-MEM or α-MEM and 10 μM dopamine for 5 min. Incubations were stopped by washing cells with ice-cold PBS, followed by the addition of sample buffer.

Receptor Internalization Assay. Immunofluorescence microscopy was used to visualize HA and Flag epitope-tagged receptors that were stably transfected into HEK-293 cells. Cells grown on glass coverslips were preincubated for 1 h with HA.11 (3 μg/mL; Berkeley Antibody Co., Richmond, CA) or M1 (3 μg/mL; Kodak, New Haven, CT) monoclonal antibody to allow the antibodies to bind the epitope-tagged receptors exposed at the plasma membrane. The experiments were stopped by fixing, and then the cells were permeabilized and visualized using fluorochrome-labeled secondary antibodies as described previously (*31*).

RESULTS

SH3 Binding Motifs within GPCR Families. Analysis of the dopamine D4 receptor sequence identified several putative SH3 binding domains conforming to the minimal conserved sequence PXXP. This motif is particularly prevalent in the polymorphic repeat and flanking sequences in the third intracellular loop (Figure 1A). A comprehensive search of the majority of the known GPCRs identified this PXXP motif in about half of these receptors. The PXXP motif is found in most of the catecholaminergic receptors. The prevalence of this motif in the catecholaminergic mammalian receptors is about 1 in 120 amino acids in the amino-terminal tail, never in the transmembrane domains, and 1 in 40 amino acids in the third intracellular loop and carboxy-terminal tail. The more stringent pΦPpXp motif (*22*) is only found in the human dopamine D4 receptor at amino acid positions 241−246 and 246−251.

Interaction of the Dopamine D4 Receptor with Nck and Grb2. Putative SH3 binding motifs can be found in the third intracellular loop of the human D4 receptor, in particular in the polymorphic repeat sequence and the immediate surrounding sequence (Figure 1A). To determine whether the third intracellular loop of the D4 receptor can interact with the SH3 domain containing proteins Nck and Grb2, we synthesized a radiolabeled fragment of the third intracellular loop of the D4.4(220−336) receptor in vitro by means of a transcription−translation system. The three SH3 domains of Nck and the entire Grb2 protein were expressed as GST fusion proteins. In the interaction assays, GST by itself did not interact with the radiolabeled receptor fragments, nor did radiolabeled luciferase control protein interact with any of the GST-SH3 fusion proteins tested (Figures 1B, 2C, 3, and 4). The third intracellular loop fragment D4.4(220−336) showed strong interaction with both the Nck and Grb2 SH3 adapter proteins (Figure 1B). Although the D4.4(220−336) fragment ran at a larger than expected size (∼30 kDa instead of ∼21 kDa), extensive sequencing revealed no out of frame mutations, indicating that this was a gel running artifact. The third intracellular loop fragment of the dopamine D4 rat receptor [D4Rat (224−305)], which does not contain the repeat sequence as seen in the human D4 receptor but does contain PXXP motifs as seen in the flanking sequences to the repeat of the human dopamine D4 receptor, was also able to interact with Nck and Grb2.

In addition to the third intracellular loop of the human D4.4 receptor, several full-length receptor products were tested for their ability to interact with Nck and Grb2 using the in vitro assay. The full-length D4.2, D4.4, and D4.7 human dopamine receptors (which contain two, four, and seven repeats, respectively) and the human dopamine D3 receptor also showed strong interaction with both Nck and Grb2 (Figure 1B). The D4.4 receptor shows a shift in relative size when immobilized with Grb2 but not with Nck, a result of the Grb2 protein running at the same relative size on the protein gel.

To determine which part of the third intracellular loop of the human D4 receptor is involved in binding to Grb2 and Nck, we made several constructs of the full-length D4.4 receptor with deletions of different parts of the third intracellular loop and a construct expressing only the two repeats (Figure 1C). The construct D4.(Δ254−315), in which almost the entire repeat sequence was deleted, maintained Nck and Grb2 interaction (Figure 1B). The interaction with D4.(Δ221−315), which has the repeat sequence and the PXXP motifs amino to the repeat sequence deleted but expresses a PXXP motif carboxy to the repeat sequence, could still interact with Nck and Grb2, but this was significantly reduced as compared to the interaction seen with the full-length D4.4 receptor. Finally, the D4.(Δ221−337) construct, in which all PXXP motifs were deleted, showed a marked reduction in interaction with both Nck and Grb2. The repeat sequence by itself [known as the αζ sequence D4.(249−280)] did not interact with Nck or Grb2.

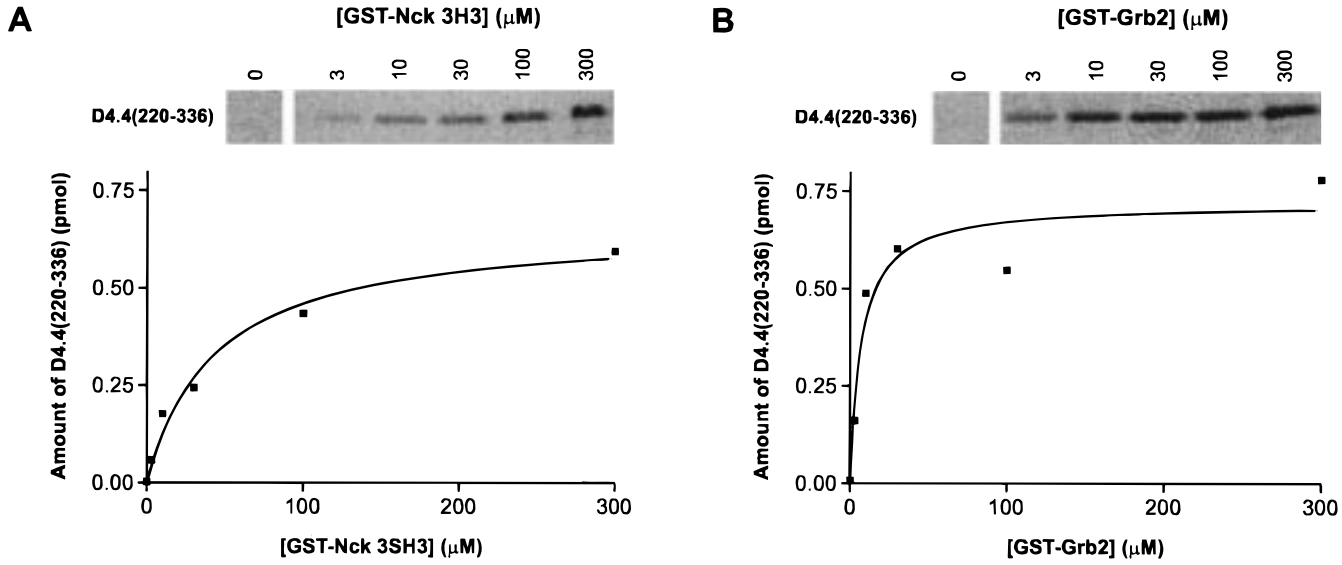
Saturation Analysis. We used a saturation analysis to quantify the strength of interaction between the D4.4 constructs and Nck or Grb2. We used increasing concentration of the GST fusion protein with a maximal concentration of 300 μM while a constant amount of in vitro labeled receptor was added. The interaction of the third intracellular loop D4.4(220−336) fragment as well as the full-length D4 constructs showed saturable binding with both Nck 3SH3 (Figure 2A) and Grb2 (Figure 2B). The calculated amount of labeled receptor in the saturation analysis (B_{max}) bound at the high concentrations of GST fusion protein was comparable to the total amount of labeled receptor added to the incubation mixture. Approximations for the affinity of the interactions were obtained from replicate experiments of the saturation analysis (Figure 2C). Nck had a K_d of about 40 μM and Grb2 had a K_d of about 10 μM for the third intracellular loop D4.4(220−336) fragment. The full-length receptors showed strong interaction with Nck 3SH3 and Grb2, although this interaction was less than that seen with the third intracellular loop by itself. Of the three receptor variants, it seems that the D4.7 displays the strongest interaction, with a K_d of about 50 μM for Grb2, while the D4.2 and D4.4 receptors have an affinity of about 200 μM for both Nck and Grb2. The deletion mutant D4.(Δ254−315), which has most of the repeat region removed, showed no reduction in binding (K_d 30−50 μM). The deletion mutant D4.(Δ221−315), which has the repeat region and the amino terminal PXXP motifs removed, has a significantly reduced interaction (K_d > 600 μM). The deletion mutant D4.(Δ221−337), which has the repeat region as well as the amino and carboxy PXXP motifs removed, displayed no binding.

Pharmacological Profile of Interaction. To determine whether the observed interaction between the SH3 domains and the D4 receptor followed the classic pharmacological rules for a specific and reversible interaction, we also performed a competition analysis with GST-Nck 3SH3 and the third intracellular loop D4.4(220−336) fragment. We competed the GST-Nck 3SH3/[^{35}Cys]D4.4(220−336) interaction with a 10-fold excess of unlabeled D4.4(220−336) or D4.(Δ254−315). The GST-Nck 3SH3/[^{35}Cys]D4.4(220−336) interaction can be significantly reduced by the addition of a 10-fold excess of unlabeled D4.4(220−336) or D4.(Δ254−315) interacting fragment prior, during, or after the interaction with [^{35}Cys]D4.4(220−336) has been established (data not shown).

Interaction of the Dopamine D4 Receptor with Other SH3 Containing Proteins. The third intracellular loop D4.4(220−336) fragment also interacted with the GST fusion proteins containing SH3 domains of Abl, Crk, p85 kinase α, and c-Src. No interaction was seen with the SH3 domain containing fragments of PLCγ and spectrin (Figure 3).

Interaction of D4.4 with Grb2 Requires both SH3 Domains for Maximal Binding. Grb2 consists of two SH3 domains separated by a single SH2 domain. The amino-terminal SH3 domain of Grb2 displayed almost no interaction with the third intracellular loop D4.4(220−336) fragment while the carboxy-terminal SH3 domain displayed only an intermediate interaction as compared to the full-length protein (Figure 4). Mutagenesis of Pro^{49} to Leu^{49}, which has been shown to abolish interactions with the amino-terminal SH3 domain (*33*), also displayed a significant reduction in binding to D4.4(220−336).

Other GPCRs Also Show an Ability to Bind Grb2 and Nck. The full-length proteins of the dopamine D3, D4, muscarinic M4, and β1 adrenergic receptors (Figures 1B and 4) could all interact with Grb2. The relative strength of the interaction



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	GST	GST-Nck 3SH3	GST-Grb2
	Kd μM	Kd μM	Kd μM
CONTROL	no binding	no binding	no binding
D4.4(220-336)	no binding	40	10
D4.2		200	200
D4.4		200	200
D4.7		200	50
D4.(Δ254-315)		30	50
D4.(Δ221-315)		>600	>600
D4.(Δ221-337)	no binding	no binding	no binding

FIGURE 2: Saturation analysis of D4.4 and various deletion mutants with Nck and Grb2. A constant amount of in vitro labeled protein (0.5 pmol) was incubated with increasing concentrations of GST-Nck 3SH3 (A) or GST-Grb2 (B). The immobilized in vitro product is shown as a scan, and the quantified results are depicted graphically. The affinity (K_d) of the various D4 constructs for Nck or Grb2, estimated from replicate experiments, are summarized (C).

between the adaptor proteins Grb2 with the receptors is D4 > D3 > M4 > β1. The muscarinic M4 and β1 adrenergic receptor showed virtually no detectable interaction with the other SH3 domain containing fusion proteins, including the carboxy-terminal Grb2 SH3 domain and the Grb2 (P49L) mutant protein.

Interaction of SH3 Domains with D4 in Saccharomyces cerevisiae. To determine if the identified SH3 binding domains in the human D4 receptor could indeed bind SH3 domain containing proteins in a cellular system, we employed the yeast two-hybrid assay. We tested whether the receptor fragments encoding the third intracellular loop of D4.2(219−304), D4.4(219−336), and D4.7(219−384) fused to the *GAL4* activation domain could interact with SH3 domains fused to the *GAL4* DNA binding domain to activate transcription of the reporter gene. All three D4 constructs showed a moderate interaction with the Nck fragment containing all three SH3 domains (data not shown). D4 did not interact with the other SH3 domain containing proteins Grb2 (N) (1−59), Grb2 (C) (158−217), Abl (65−123), PLCγ (792−851), and Crk (235−293). The full-length Grb2 protein construct resulted in self-activation and therefore could not be tested for its ability to interact with D4. The number of repeats did not result in clear detectable differences in the relative strength of interaction. Strong interactions were seen in the positive control group testing the established Sos/Grb2 and Sos/Nck interaction as well as for Grb2/Crk and Abl/Crk (*30, 34, 35*).

Pharmacological Characterization of Deletion Mutants. Saturation binding analysis with [^{3}H]spiperone indicated that the D4.7 receptor and the two deletion mutant receptors D4.(Δ221−315) and D4.(Δ221−337) showed saturable, concentration-dependent binding with affinities in the range of 100−300 pM and a B_{max} of about 2 pmol/mg of protein. Competition of [^{3}H]spiperone binding by dopamine indicated no significant differences between the D4.7 receptor and the deletion mutant receptors D4.(Δ221−315) and D4.(Δ221−337). All receptors showed a high affinity for dopamine binding in the order of 1−5 nM, which could be shifted by Gpp(NH)p to a lower affinity (Figure 5A). Cells expressing only the expression vector pcDNA3 displayed no measurable [^{3}H]spiperone binding.

GTPγ^{35}S Binding of the Deletion Mutant D4 Receptors. Functional coupling of the dopamine D4.7, D4.(Δ221−315), and D4.(Δ221−337) receptors was measured by dopamine-induced GTPγ^{35}S binding. These binding assays demonstrated a concentration-dependent dopamine-mediated binding of GTPγ^{35}S to these receptors (Figure 5B). Cells expressing only the expression vector pcDNA3 displayed no measurable induction of GTPγ^{35}S binding by dopamine.

Modulation of Intracellular cAMP Levels by Mutant D4 Receptors. Intracellular cAMP levels were measured in CHO-K1 cells expressing either the D4.7 receptor or the deletion mutant D4.(Δ221−315) or D4.(Δ221−337). Forskolin-stimulated increases in cAMP levels were blocked by dopamine in cells expressing the D4.7 receptor but not in

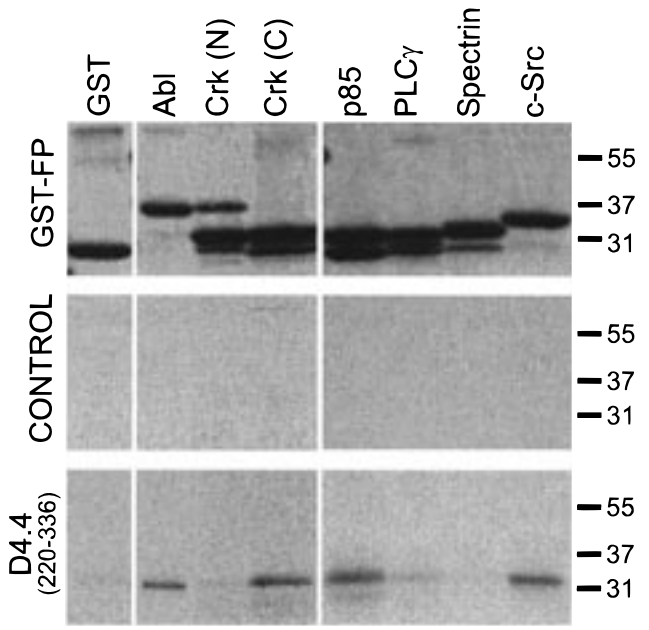

FIGURE 3: In vitro interaction of D4 with various SH3 domain containing proteins. In vitro ^{35}Cys-labeled D4.4(220−336) protein (0.5 pmol) was incubated with equivalent amounts of various GST fusion proteins (∼30 μM) expressing either the whole protein or only the SH3 domain containing fragments of various SH3 domain containing proteins (see Experimental Procedures for abbreviations). The top panel shows the relative amounts of GST fusion protein (Brilliant Blue Stain) used to precipitate the different in vitro labeled proteins (96 h exposure) shown in the lower panels. Molecular masses are indicated in kilodaltons.

cells expressing the deletion mutant receptor D4.(Δ221−337) (Figure 5C). Stimulation of the D4.(Δ221−315) deletion mutant with dopamine showed a decreased ability to inhibit forskolin stimulated cAMP levels.

Activation of MAPK Pathway. A MAPK assay was performed to assess the ability of the D4 receptor and D4 deletion mutants to activate the MAPK pathway. Stimulation of the D4.7 receptor in CHO-K1 cells by dopamine (10 μM) resulted in strong activation of the MAPK pathway as determined by the immunodetection of the phosphorylated Erk1 and Erk2 MAP kinases (Figure 5D). The deletion mutants D4.(Δ221−315) and D4.(Δ221−337) were unable to activate the MAPK pathway.

Internalization of D4.4 and the D4.4 Deletion Mutants. The internalization of D4.7 and the deletion mutants D4.(Δ221−315) and D4.(Δ221−337) was analyzed using an antibody uptake experiment in untreated cells. Plasma membrane bound receptor was first labeled with a primary antibody, and then after the cells were incubated, the internalization of the receptor−antibody complex was visualized by immunofluorescence. The D4.7 receptor was localized predominantly in the plasma membrane (Figure 6A) while the deletion mutant that removed all the potential SH3 binding domains, D4.(Δ221−337), was predominantly localized in endosomal vesicles (Figure 6C). The D4.(Δ221−315) deletion mutant, which still contains a potential SH3 binding domain carboxy to the repeat region, showed an intermediate phenotype for internalization (Figure 6B).

DISCUSSION

We have shown that all of the GPCRs that we tested in this study (including the dopamine D3 and D4 receptors,

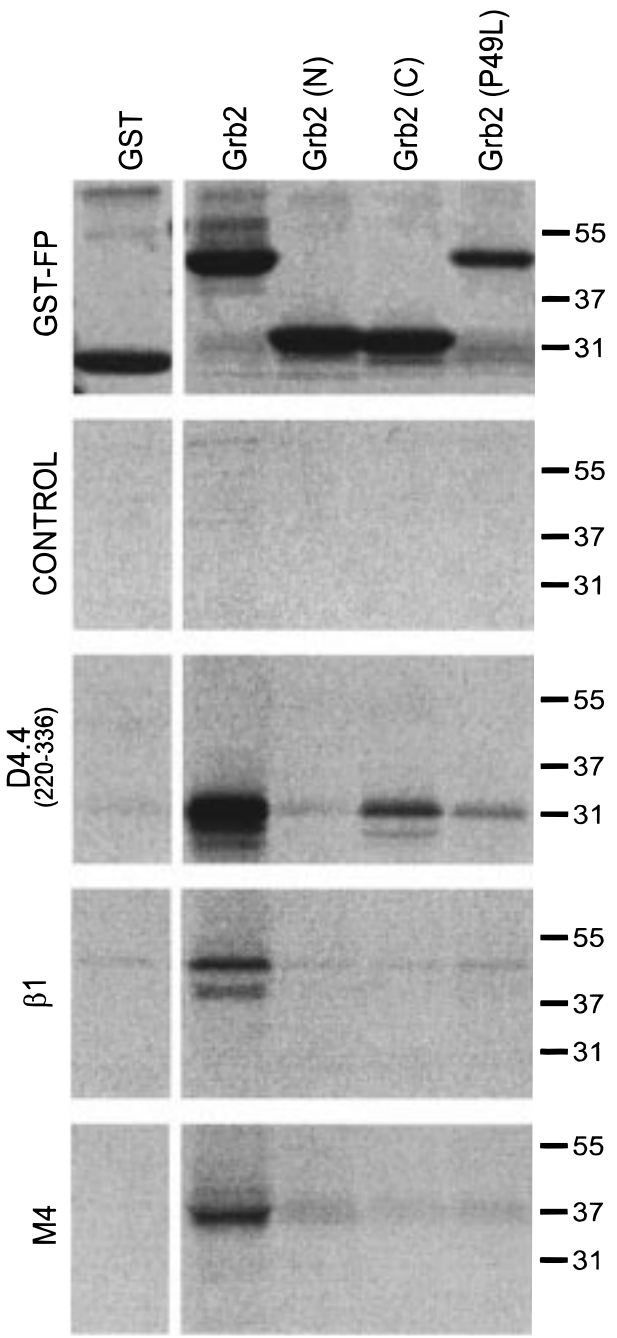

FIGURE 4: In vitro interaction of GPCRs with different Grb2 SH3 domains. In vitro ^{35}Cys-labeled D4.4(220−336), β-adrenergic β1 receptor, muscarinic M4 receptor, and control (luciferase) proteins (0.5 pmol) were incubated with equivalent amounts of various GST fusion proteins (∼30 μM) expressing either the whole protein or only the SH3 domain containing fragments of Grb2 (see Experimental Procedures for abbreviations). The top panel shows the relative amounts of GST fusion protein (Brilliant Blue Stain) used to precipitate the different in vitro labeled proteins (96 h exposure) shown in the lower panels. Molecular masses are indicated in kilodaltons.

the β1 adrenergic receptor, and the muscarinic M4 receptor) showed moderate to strong interaction in vitro with at least one of the tested SH3 domain containing proteins. The D4 receptor interacts in vitro with the SH3 domains of Grb2, Nck, Abl, Crk, p85α, and c-Src but not with PLCγ and spectrin. An interaction with Grb2 and Nck was also observed with the D3 dopamine receptor. The interaction of the β1 adrenergic and M4 muscarinic receptors with these

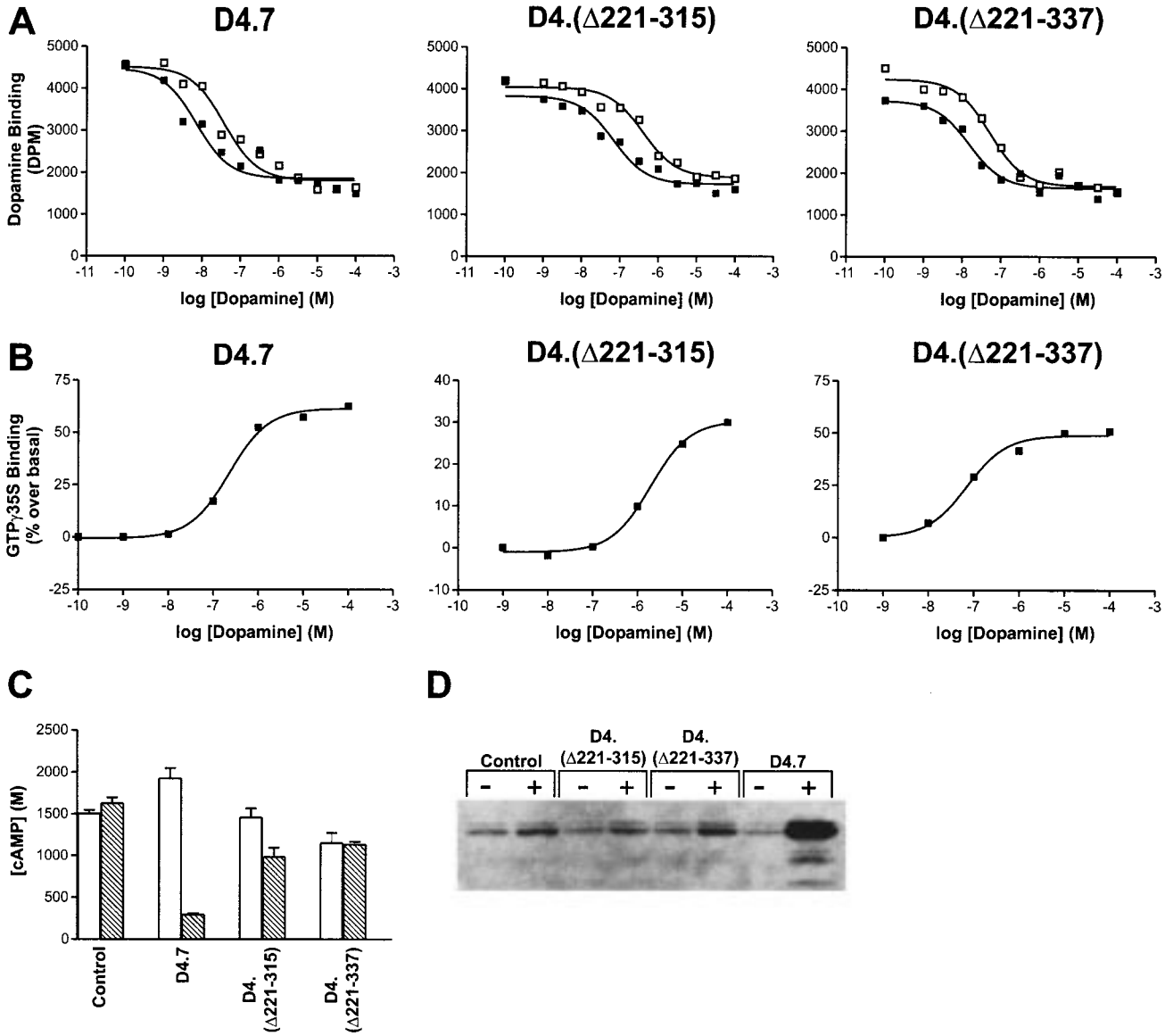

FIGURE 5: Functional characterization of the D4.7 receptor and the D4 deletion mutant receptors. (A) Competition binding analysis of [^{3}H]spiperone binding on membranes isolated from the cell lines D4.7, D4.(△221−315), or D4.(△221−337) by dopamine in the presence (□) and absence (■) of Gpp(NH)p was done as described in the Experimental Procedures. The logarithm of the concentration of the drug in molar (M) used is indicated on the *x*-axis. The amount of specific binding expressed as disintegrations per minute (dpm) is indicated on the *y*-axis. The assay was done in parallel for the different receptors in the presence and absence of Gpp(NH)p using the same membranes preparations. The results are representative of two independent experiments. (B) Dopamine stimulated GTPγ^{35}S binding by the D4.7 and deletion mutant receptors is expressed as percent over basal binding. The logarithm of the concentration of the drug in molar (M) used is indicated on the *x*-axis. The amount of GTPγ^{35}S binding expressed as percent over basal binding is indicated on the *y*-axis. The results are representative of two independent experiments. (C) Inhibition of forskolin-stimulated cAMP levels by dopamine in CHO-K1 stable cell lines expressing the D4.7 or the deletion mutant receptors is shown. Intracellular levels of cAMP (M) was measured after incubation of cells with forskolin (10 μM) in the presence (hashed bar) or absence (open bar) of dopamine (10 μM) for 30 min. The average of three independent experiments assayed in duplicate are shown. The CHO-K1 cell line expressing pcDNA3 was used as control. (D) Immunoblot detection of the phosphorylated Erk1 or Erk2 MAPK proteins after stimulation of the dopamine receptors or deletion mutants with dopamine (10 μM) in CHO-K1 cells. In all of these experiments, the cells stably expressing the eukaryotic vector pcDNA3 in CHO-K1 cells served as control. The blot is representative of three independent experiments. The CHO-K1 cell line expressing pcDNA3 was used as control.

proteins appears to be restricted to the adapter proteins Grb2 and Nck. This indicates that receptor interactions with SH3−SH2 adaptor proteins are not restricted to the dopaminergic receptor family and may serve a more general role in GPCR function.

Specificity of the in vitro interaction is highlighted by the fact that the luciferase control protein, which contains a PXXP motif, does not interact with any of the SH3 domains tested. In addition, the different GPCRs interacted only with a subset of the SH3 domains tested, and not every SH3 domain of Grb2, Crk, and Nck could interact with the receptors. Furthermore, the binding of Nck and Grb2 to D4 demonstrates specific, saturable, and reversible binding.

The different full-length human D4 receptors (D4.2, D4.4, and D4.7) seem to bind with a somewhat lower affinity to Nck and Grb2 as compared to the construct expressing only the third intracellular loop [D4.4(220−336)]. The D4.7 receptor as well as the D4.(Δ254−315) deletion mutant seem

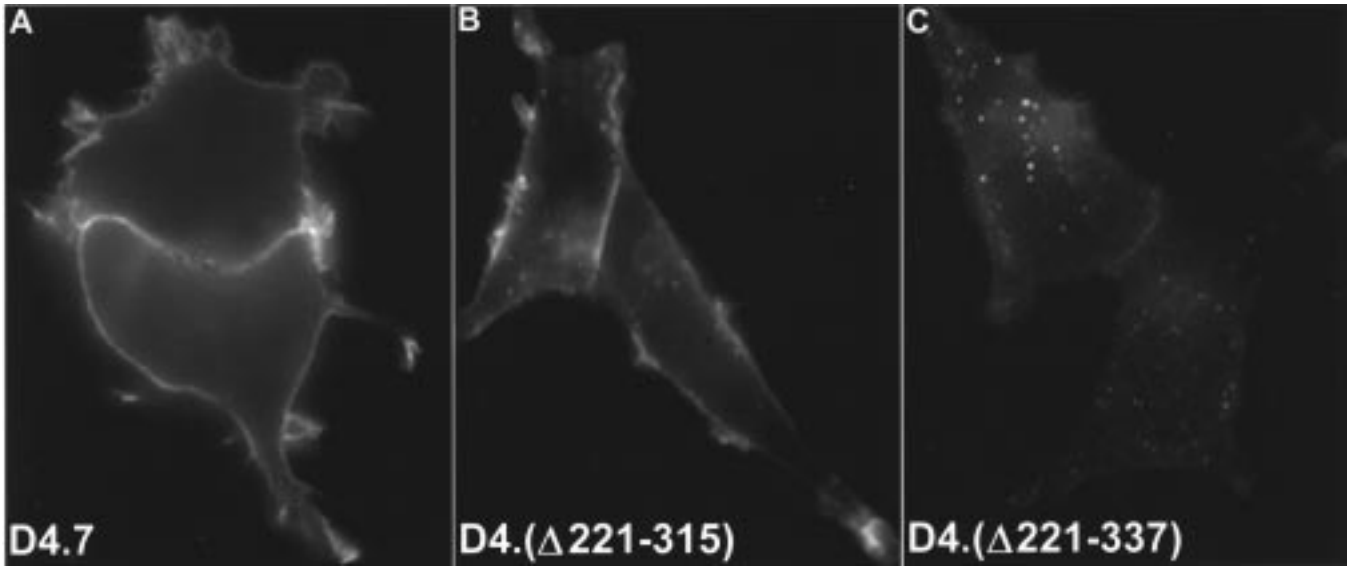

FIGURE 6: Receptor internalization assay. Immunofluorescence microscopy was used to visualize Flag epitope-tagged versions of the D4.7 (A), D4.(△221−315) (B), and D4.(△221−337) (C) receptors that were transiently transfected into HEK-293 cells. The subcellular distribution of the receptors were visualized using flurochrome-labeled secondary antibody (see Experimental Procedures for details). The HA epitope-tagged receptors showed a similar distribution (data not shown).

to bind with a stronger affinity to Grb2 as compared to the D4.2 and D4.4 receptors. The binding of the different D4 receptors and the deletion mutants displays a similar binding profile for both Nck and Grb2. The rat D4 receptor third intracellular loop fragment that does not exhibit repeats but that contains several potential SH3 binding sites as seen in the flanking region of the human D4 repeat was also able to bind to Nck and Grb2. Furthermore, the D4.2 repeat sequence, known as $\alpha\zeta$, did not interact with Nck or Grb2. This suggests that the third intracellular loop is sufficient for maximal binding and that the repeats themselves are not essential for this interaction but may modulate the strength of the interaction.

Deletion analysis of the D4 receptor indicated that Grb2 and the fragment of Nck containing the three SH3 domains interact with binding domains immediately upstream and downstream from the repeat sequence in the D4 receptor but not with the repeat sequence itself. These deletion analysis studies suggest that the interaction is cooperative between these two regions in the D4 receptor with the adapter proteins since D4.4(Δ254−315), which lacks the repeat, displays a strong interaction (K_d of 40 μM) while D4.(Δ221−315), which lacks the repeat and amino PXXP motifs, has a significant reduced interaction (K_d > 600 μM), which is further diminished by consecutive deletion of the fragment containing the PXXP motif carboxy to the repeat [D4.(Δ221−337); no binding].

By examining the interaction of the D4 receptor with the amino-terminal SH3 domain as compared to the carboxy-terminal domain of Grb2, it was found that the carboxy SH3 domain fragment is the primary site of interaction, although the whole protein shows a significantly enhanced interaction. The amino SH3 domain by itself showed no interaction, but mutagenesis of Pro^{49} to Leu^{49} in the amino-terminal SH3 domain shown to disrupt SH3 binding (*33*) significantly reduced the interaction of the whole protein with the D4 receptor, indicating that the interaction with Grb2 with the D4 receptor is of a cooperative nature between the amino-terminal and the carboxy-terminal SH3 domains. Since only the full-length Grb2 protein appears to interact with the muscarinic M4 and β1 adrenergic receptor, Grb2 may also interact in a cooperative manner with these receptors. This type of cooperative interaction has been previously demonstrated for Grb2 binding to HPK1 (*29*). Our D4 deletion studies together with the observed differential interactions between the D4 and the different Grb2 fragments and mutant strongly indicate that the different SH3 domains of Grb2 interact in a cooperative manner with the two SH3 binding domains upstream and downstream from the repeat sequence in the D4 receptor. Our data indicate that the absence of SH3 domain mediated interaction using single SH3 domains should be interpreted with caution with respect to their involvement in the binding of the holoprotein.

An interaction in cells was demonstrated by expressing the D4 interacting fragment and the Nck SH3 domains as fusion proteins with the *GAL4* activator and DNA binding proteins in yeast. The yeast two-hybrid system has been successfully used to determine several SH3 interactions with good predictive capabilities that subsequently have been confirmed in vivo (*30, 36*). Although Nck showed an interaction in this system, the amino or carboxy fragment of Grb2 failed to interact with the D4 receptor. The failure of Grb2 to interact is not due to a lack of expression since we could successfully demonstrate an interaction in yeast between Grb2 and the Sos tail or the Crk SH2 domain. Furthermore, western analysis demonstrated approximately equivalent levels of expression of Grb2 and Nck fusion proteins (data not shown). We cannot exclude that the conformation of the Grb2−DNA binding and D4.4(219−336)−activator complex may have interfered with the ability of this complex to bind to or activate the *GAL4* promoter of the reporter construct. Alternatively, it is possible that Grb2 failed to show an interaction because we only used the amino- or carboxy-terminal SH3 domains of the Grb2 fusion constructs, which were shown in vitro to have a significantly reduced

interaction with the D4 receptor. Attempts at expressing the entire Grb2 protein in yeast resulted in self-activation, thus preventing us from observing an interaction with D4. The data are consistent with the interpretation that the D4-SH3 interaction is of a cooperative nature between two distinct SH3 domains and their binding sites in the D4 receptor.

The functional importance of the identified SH3 binding domains in the D4 receptor was explored by the disruption of these domains in the amino-terminal epitope-tagged receptor. Functionally and pharmacologically, the wild-type D4 receptor was indistinguishable from both the HA and Flag epitope-tagged versions of the receptor. With respect to receptor binding, we demonstrated that both spiperone binding and the binding of dopamine is unaffected by the deletion of the putative SH3 binding domains. Inclusion of the non-hydrolyzable GTP analogue Gpp(NH)p in the binding assays resulted in a decreased binding affinity for dopamine. In addition, dopamine can stimulate GTP−GDP exchange for both the wild-type and mutant D4 receptors as measured by a GTPγ^{35}S binding assay. This indicated that the receptor has retained the ability to interact functionally with G proteins. Nevertheless, the deletion of the putative SH3 binding domains resulted in disruption of functional coupling of the activated receptor to adenylyl cyclase and MAPK.

Disruption of the SH3 binding domain affected receptor internalization. Internalization was studied by an immunofluorescence detection protocol that allowed the detection of receptors prelabeled with a primary antibody on the extracellular domain of the cell (*31*). Following a 30-min incubation period, in the absence of receptor stimulation, the receptors were detected with a secondary immunofluorescent antibody. This way it was shown that the SH3 binding domain deleted receptors were predominantly localized in endosomal vesicles, suggesting a strong constitutively internalized phenotype of these receptors. The constitutively internalized phenotype may explain the inability of the receptor to show significant coupling to the second messenger pathways. This is further supported by the deletion mutant that still contains the carboxy SH3 binding domain, which shows an intermediate phenotype for internalization and also shows a moderate ability to couple to adenylyl cyclase. The deletion mutant D4.(Δ254−315), which does not express the repeat, is still functionally active (*17, 19*) and displays no internalization deficit (not shown), suggesting that the repeat sequence itself is not involved in the interaction deficit.

In conclusion, we have shown that the dopamine D4 receptor and possibly several other GPCRs can interact in vitro with SH3 domain containing proteins, in particular with the adapter proteins Nck and Grb2. This interaction appears to be mediated through a high-affinity cooperative binding between two distinct SH3 domains and their binding sites. Deletion of the putative SH3 binding domains results in a receptor that still can bind dopamine, couple to G-protein, but has lost its ability to stimulate MAPK and inhibit forskolin-stimulated cAMP levels. Furthermore, the deletion mutant receptor is constitutively internalized. These results suggest that GPCRs, in particular the dopamine D4 receptor, may interact with SH3 binding domains and that these domains fall within the region of the receptor that is involved in receptor internalization.

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