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Modulation of Dopamine D_2 Receptor Signaling by Actin-Binding Protein (ABP-280)

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ABSTRACT

Proteins that bind to G protein-coupled receptors have recently been identified as regulators of receptor anchoring and signaling. In this study, actin-binding protein 280 (ABP-280), a widely expressed cytoskeleton-associated protein that plays an important role in regulating cell morphology and motility, was found to associate with the third cytoplasmic loop of dopamine D_2 receptors. The specificity of this interaction was originally identified in a yeast two-hybrid screen and confirmed by protein binding. The functional significance of the D_2 receptor-ABP-280 association was evaluated in human melanoma cells lacking ABP-280. D_2 receptor agonists were less potent in inhibiting forskolin-stimulated cAMP production in these cells. Maximal inhibitory responses of D_2 receptor activation were also reduced. Further yeast two-hybrid experiments showed that ABP-280 association is critically dependent on the carboxyl domain of the D_2 receptor third cytoplasmic loop, where there is a potential serine phosphorylation site (S358). Serine 358 was replaced with aspartic acid to mimic the effects of receptor phosphorylation. This mutant (D_2S358D) displayed compromised binding to ABP-280 and coupling to adenylate cyclase. PKC activation also generated D_2 receptor signaling attenuation, but only in ABP-containing cells, suggesting a PKC regulatory role in D_2-ABP association. A mechanism for these results may be derived from a role of ABP-280 in the clustering of D_2 receptors, as determined by immunocytochemical analysis in ABP-deficient and replete cells. Our results suggest a new molecular mechanism of modulating D_2 receptor signaling by cytoskeletal protein interaction.

Dopamine D_2 receptors, which belong to the superfamily of G protein-coupled receptors (GPCRs), regulate essential neurobiological and endocrine processes. Hence, the cellular signaling of D_2 receptors has been extensively investigated. Beyond inhibiting adenylate cyclase, D_2 receptors modulate the activities of potassium and calcium channels, phospholipase C, mitogen-activated protein kinase, sodium-proton exchangers, and the release of arachidonic acid (Sibley and Monsma, 1992; Civelli et al., 1993; Neve and Neve, 1997; Missale et al., 1998). These D_2 signaling pathways seem to be regulated in a complex fashion. For instance, protein kinase C (PKC) phosphorylation directs the preferential coupling of D_2 receptors from the inhibition of adenylate cyclase to the release of arachidonic acid (Di Marzo et al., 1993). Cell type specificity is exhibited as D_2 receptors inhibit phospholipase C in pituitary cells, but activate it in fibroblast cells (Vallar et al., 1990). The effector coupling efficiency of D_2 receptors seems also to be regulated by the subcellular localization of these receptors. In particular, presynaptic D_2 autoreceptors found on the axonal terminals of dopaminergic neurons are more sensitive to agonist stimulation than their identical postsynaptic counterparts (Skirboll et al., 1979; Clark and White, 1987; Missale et al., 1998). In light of these differences in both the cell-type specificity and coupling efficiency of D_2 receptors, cellular pathways additional to G protein coupling are likely to be involved in the activity and regulation of D_2 receptor signaling.

Effector coupling and membrane targeting of GPCRs have been found to be regulated by a variety of protein-protein interactions. A nearly universal mechanism of terminating GPCR signaling is mediated by the binding of arrestins after receptor phosphorylation by GPCR kinases (Krupnick and Benovic, 1998). Recently, proteins that bind to specific members of GPCRs have been identified as unique players in receptor signaling or targeting. For example, the association of β_2 adrenergic receptors with the protein translation initiation factor (eIF-2B) has been shown to enhance the ability of these receptors to activate adenylate cyclase (Klein et al., 1997). β_2 adrenergic receptors also activate sodium-proton exchangers by recruiting regulatory factors in an agonist-dependent but G protein-independent fashion, indicating the

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ABBREVIATIONS: GPCR, G protein coupled receptor; PKC, protein kinase C; ABP-280, actin-binding protein 280; MBP, maltose-binding fusion protein; GST, glutathione *S*-transferase; CHO, Chinese hamster ovary; TBS-T, Tris-buffered saline/Tween 20; TEM, Tris/EDTA/$MgCl_2$; 6,7-ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide; PMA, 4-β-phorbol-12-myristate-13-acetate; FITC, fluorescein isothiocyanate; PDZ, PSD-95/DlgA/ZO-1.

existence of novel signaling mechanisms distinct from traditional GPCR second messenger pathways (Hall et al., 1998). Moreover, a family of single-transmembrane-domain proteins has been identified as modifying proteins for calcitonin-receptor-like receptors (McLatchie et al., 1998). These single-transmembrane-domain proteins are required for the targeting of calcitonin-receptor-like receptors to the plasma membrane and also determine their ligand specificity. Finally, ATRAP, a novel protein that interacts with the carboxyl-terminal cytoplasmic domain of the angiotensin II type 1 receptor has been found to negatively regulate receptor signaling (Daviet et al., 1999).

We have investigated the possibility that novel protein interactions with the D_2 receptor may regulate its signaling or targeting. Here, we show that cytoskeletal protein actin-binding protein 280 (ABP-280) interacts with the third cytoplasmic loop of the D_2 receptor. We demonstrate that this association enhances coupling efficiency of D_2 receptors to adenylate cyclase, can be regulated by PKC activation, and seems to play a role in cell surface receptor clustering.

Materials and Methods

Yeast Two-Hybrid. The third cytoplasmic loop of the human D_2 receptor (residues 211–372) was amplified by PCR and subcloned in-frame into the Gal4 DNA-binding domain vector pGBT9 (Clontech, Palo Alto, CA) to generate pGBT9-D_2. PGBT9-D_2 was used to screen a human brain cDNA library constructed in the Gal4 activation domain vector pACT2 (Clontech). Library plasmid DNAs were isolated by plating 5×10^6 independent clones on 80 150-mm LBA plates. Handling and transformation of yeast (strain Y190) were performed as described (Matchmaker Two-hybrid System protocol; Clontech). Briefly, yeast cells were sequentially transformed with pGBT9-D_2 and 300 μg of library plasmid DNA using the lithium acetate method. The final transformation mixture (2.6×10^6 yeast plasmid transformants) was plated onto 100 150-mm synthetic dextrose agar plates lacking tryptophan, leucine, and histidine but containing 20 mM 3-aminotriazole and allowed to grow for a week at 30°C. Robust colonies were restreaked on fresh plates and tested for β-galactosidase activity with X-gal as a substrate. Positive clones were grown-up in liquid yeast media and library plasmids were recovered and sequenced. The intensity of protein-protein interactions were quantified using *o*-nitrophenyl-β-D galactopyranoside (Sigma, St. Louis, MO) as a substrate.

In Vitro Protein-Protein Binding. The third cytoplasmic loops of D_1, D_2, D_3, and D_4 receptors were subcloned in frame into bacterial expression vector pGEX-3X (Pharmacia, Piscataway, NJ) to generate glutathione *S*-transferase (GST) fusion proteins (note: use of the D_4 receptor in all experiments was limited to the $D_{4.4}$ isoform). A single bacterial colony was used to inoculate 2× YTA medium and incubated at 30°C until an A_{600} of 0.3 was reached. Isopropyl -β-thiogalactopyranoside (30 μM) was then added to induce the formation of fusion proteins. Bacteria were pelleted at 5,000 g for 5 min, resuspended in PBS, and lysed by sonication. After centrifugation twice at 13,000 g for 15 min at 4°C, supernatant was collected as the bacterial lysate. ABP-280 (residues 1779–2143) was purified as a maltose-binding fusion protein (MBP) with amylose affinity chromatography (New England Biolabs, Beverly, MA). An aliquot of amylose resin was added to the bacterial supernatant prepared by sonication. After a 30-min incubation at room temperature, the amylose resin was collected by centrifugation at 1,000 g for 1 min. The pelleted resin was then washed five times with PBS and used as an immobilized MBP-ABP280 fusion protein. For in vitro protein-protein binding, bacterial lysates containing GST-D_1, GST-D_2, GST-D_3, and GST-D_4 were incubated with immobilized MBP-ABP280 at 4°C overnight in PBS. The amylose resin was spun down and washed with PBS three times. Resin-bound proteins were then eluted with 0.5 M maltose and visualized by Western blotting with mouse GST monoclonal antibody (Santa Cruz Biotech, Santa Cruz, CA). For Western blot analysis, proteins were electrophoresed through SDS-polyacrylamide gel and transferred to nitrocellulose filters. Filters were rinsed with Tris-buffered saline/Tween 20 (TBS-T) buffer (20 mM Tris · HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20), blocked with 5% milk and 3% BSA in TBS-T for 1 h at room temperature. Proteins of interest were then identified by incubating the blot with the mouse glutathione *S*-transferase (GST) monoclonal antibody, followed by washing in TBS-T and incubating with HRP-conjugated antimouse IgG antibody in TBS-T. After extensive washing, the signal was detected by enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

Cell Culture and Transfection. ABP-280 is ubiquitously expressed in different types of cells and tissues (Takafuta et al., 1998). Western blot analyses revealed that ABP-280 is expressed in Chinese hamster ovary (CHO), human embryonic kidney 293 and Ltk^-, cell lines that are commonly used to express D_2 receptors heterologously (data not shown). A human melanoma cell line (M_2) that does not express ABP-280 endogenously has been described (Cunningham et al., 1992). The A_7 cell line, a M_2 subclone that has been stably transfected with ABP-280 cDNA (Cunningham et al., 1992), was used as a control. As assessed by the ratio of actin and ABP-280, the expression of ABP-280 in A_7 cells is comparable with most cell lines. M_2 and A_7 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum and penicillin. CHO cells and human embryonic kidney 293 cells were grown in α-minimal essential medium and Dulbecco's modified Eagle's medium, respectively with the similar supplements. Stably transfected CHO, A_7, and M_2 cells were selected with either G418 (500 μg/ml; Life Technologies, Grand Island, NY), or zeocin (100 μg/ml; InVitrogen, San Diego, CA) for 2 weeks. Clones surviving the selections were expanded and analyzed by receptor radioligand binding or cAMP assay.

Receptor Radioligand Binding. Stably transfected cells were grown to confluency on 150-mm tissue culture plates. Cells were rinsed with 5 ml of ice-cold PBS once and scraped off the plates in the TEM buffer (25 mM Tris · HCl, pH 7.4, 1 mM EDTA, and 6 mM $MgCl_2$). After centrifugation at 1200 g for 3 min at 4°C, cell pellets were frozen at −70°C until the day of assay. Cell pellets were resuspended in ice-cold TEM buffer at a concentration of 200 to 400 μg of total protein/ml and homogenized using a polytron homogenizer (Brinkmann Instruments, Westburg, NY) at a setting of 5 for 10 sec on ice. Radioligand binding was performed in a volume of 1 ml using approximately 200 μg of total protein per tube. Competition binding assays were performed using 0.1 to 0.3 nM [^{3}H]spiperone (99 Ci/mmol; Amersham) and various concentrations of competing compounds. Reaction mixtures were then incubated for 1 h at room temperature and terminated by rapid vacuum filtration through GF/B filters presoaked in 0.5% polyethylenimine (Sigma) using a 24-port harvester (Brandel, Montreal, Canada). Filters were rapidly washed with 5 ml of TEM buffer, air dried, and individual filter discs were placed in counting vials with 5 ml of scintillation fluid for counting in a Beckman LS-6800 liquid scintillation counter. Data were analyzed using GraphPad software (San Diego, CA).

Receptor Functional Analyses. Stably transfected M_2 and A_7 cells with similar expression levels of D_2 receptors were selected. For cAMP assay, cells were seeded 24 h before the assay at a density of 1×10^5 cells/well. Cells were washed with warm HBBS buffer (20 mM HEPES, pH 7.2, 118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM D-glucose) and incubated for 10 min at 37°C in 2 μM Ro 20–1724 to inhibit cAMP phosphodiesterase. All the cells were stimulated with 10 μM forskolin and increasing concentration of dopamine or (±)-2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (6,7-ADTN; RBI, Natick, MA). For PKC activation experiments, cells were incubated with forskolin (10 μM) and increasing concentrations of 6,7-ADTN in the presence of 100 nM 4-β-phorbol-12-myristate-13-acetate (PMA; Sigma). Drug incubation

was carried out at 37°C for 30 min and terminated by the addition of ice-cold 70% ethanol. cAMP samples were collected in Eppendorf tubes. After drying down, cAMP levels in the samples were determined using a sensitive succinylation method. Each cAMP sample was dissolved in 1 ml of ice-cold NaOAc (50 mM, pH 6.2). One hundred microliters of the sample were succinylated by incubating with 15 mM succinic anhydride (dissolved in 25% triethylamine/75% acetone) on ice for 10 min. Succinylation was terminated by the addition of 2 ml of ice-cold NaOAc (50 mM). One hundred microliters of the dilute succinylated sample were incubated with 100 μl anti-cAMP antibody (Sigma) for 4 h at 4°C. An aliquot of ^{125}I-cAMP (0.0045 μCi) (NEN, Boston, MA) was added into each sample. After overnight incubation at 4°C, immunocomplexes were precipitated with 100 μl of 10% bovine serum albumin and 100 μl of 95% ice-cold ethanol. Radioactivity was determined by counting in a Beckman 5500 Gamma counter. This succinylation radioimmunoassay has a detection limit of 100 fmol cAMP. Data were analyzed using GraphPad software.

TABLE 1

Protein-protein interactions detected with the yeast two-hybrid assay

Yeast strain Y190 cells were cotransformed with expression vectors pACT2 and pGBT9 encoding either the third cytoplasmic loops from various dopamine receptors or ABP-280 (1779-2134). Controls for protein expression include a consistently observed growth of transformed yeast on synthetic dextrose plates lacking essential amino acids, tryptophan and leucine, which are encoded by each plasmid. HIS3 indicates yeast colony growth on plates lacking tryptophan, leucine, and histidine. β-Gal indicates a color reaction of β-galactosidase filter assay. The intensity of protein-protein interaction was quantified using ONPG (*o*-nitrophenyl β-D galactopyranoside) as a substrate. The numbers shown in the ONPG assay are the arbitrary units of β-galactosidase activity.

	HIS3	β-Gal	ONPG
pACT2-ABP:pGBT9-D_2 (211–372)	++	++	65 ± 8.7 (3)
pGBT9-ABP: pACT2-D_2 (211–372)	++	++	
pACT2-ABP: pGBT9-D_{2s} (211–343)	++	++	
pACT2-ABP: pGBT9-$D_2\Delta$314–368	–	–	
pACT2-ABP: pGBT9-D_3 (210–375)	+++	+++	93
pGBT9-ABP: pACT2-D_3 (210–375)	+++	+++	
pACT2-ABP: pGBT9-$D_{4.4}$ (214–346)	–	–	0.06
pACT2-ABP: pGBT9-D_1 (215–272)	–	–	0.1
pACT2-ABP: pGBT9	–	–	
pACT2-ABP: pGBT9-D_2S358D	+	+	34 ± 6.1 (3)*

ONPG, *o*-nitrophenyl β-D-galactopyranoside.
* $P < .05$, D_2S358D versus D_2, unpaired Student's *t* test.

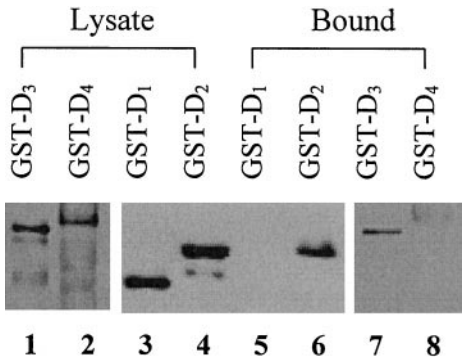

Fig. 1. Binding of ABP-280 to GST-D_2 in vitro. Bacterial lysates containing GST-D_1, GST-D_2, GST-D_3, and GST-D_4 fusion proteins were tested for their abilities to bind to purified MBP-ABP280. Lanes 1 to 4 indicate GST fusion proteins from bacterial lysates (Lysate). Lanes 5 to 8 indicate GST fusion proteins retained after incubation with MBP-ABP280 (Bound). GST-D_2 and GST-D_3, but not GST-D_1 or GST-D_4, readily bound to MBP-ABP280.

TABLE 2

Ligand affinity of dopamine D_2 receptor in M_2, A_7, and CHO cells

K_i values (nM) were calculated from [^{3}H]spiperone (0.1–0.3 nM) competition binding of agonists and antagonists on D_2 stably expressed M_2 (ABP-280-deficient), A_7 (genetically reconstituted ABP-280), and CHO cells. Curves were fitted as one-site competition binding. Data shown are the mean ± S.E. of three independent experiments, assayed in duplicate. D_2 receptor expression levels were 2.48 pmol/mg of protein (M_2) versus 2.55 pmol/mg of protein (A_7). The stable CHO cell clone used contained receptor levels of 1.07 pmol/mg of protein. No significant change in either agonist or antagonist affinity for D_2 receptors was observed in M_2, A_7, or CHO cells.

	A_7-D_2	M_2-D_2	CHO-D_2
Dopamine	8,900 ± 2,630	10,100 ± 4,970	5,330 ± 2,020
Dopamine + GTP*	25,175	26,455	ND
6,7-ADTN	350 ± 70	330 ± 120	ND
(−)-Sulpiride	170 ± 40	160 ± 10	ND
Haloperidol	3.16 ± 0.29	2.41 ± 0.47	1.80 ± 0.60

*GTP concentration was 50 μM; K_i was calculated as the average of two experiments.
ND, not determined.

Immunocytochemistry. A_7 and M_2 melanoma cells grown in 35-mm glass-bottomed culture dishes (MatTek, Ashland, MA) were transiently transfected with amino-terminally FLAG-tagged D_2 or D_1 receptors. Two days after transfection, cells were washed in PBS and subsequently fixed in 3.7% formaldehyde in PBS for 30 min on ice. After three washes with PBS, cells were blocked in 5%BSA for

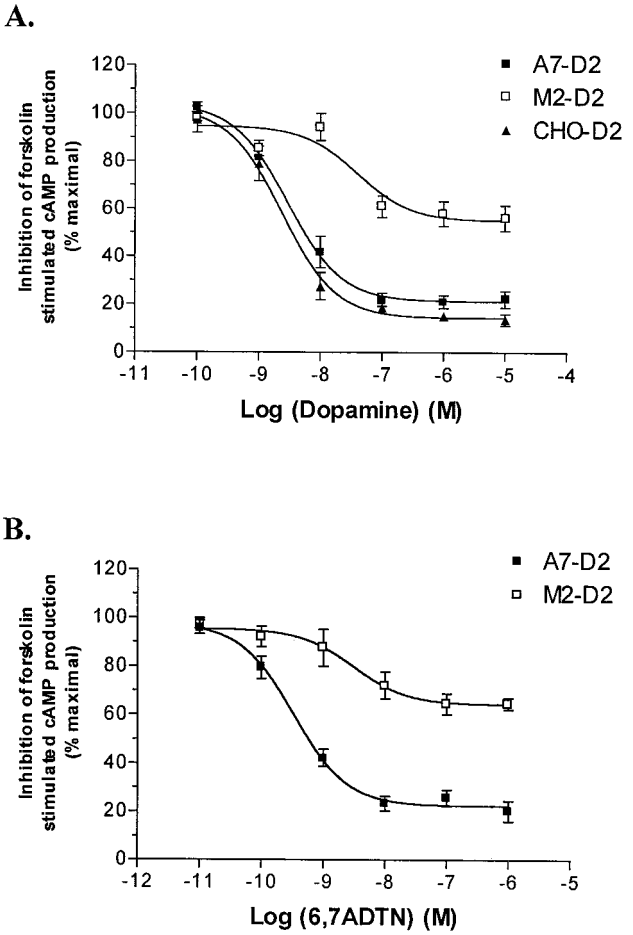

Fig. 2. D_2 receptor-mediated inhibition of cAMP accumulation in M_2, A_7, and CHO cells. Dose-response curves for (A) dopamine or (B) 6,7-ADTN inhibition of cAMP accumulation were determined on cells stimulated with 10 μM forskolin and increasing concentrations of dopamine or 6,7-ADTN. Results are expressed as percentage inhibition of forskolin-stimulated cAMP level. Curves are plotted as average ± S.E. from three to five independent experiments. D_2 receptor expression levels were 2.48 pmol/mg protein (M_2) versus 2.55 pmol/mg protein (A_7). The stable CHO cell clone used contained receptor levels of 1.07 pmol/mg protein. The typical cAMP level (per well) in the presence of 10 μM forskolin is 15.0 pmol for M_2 cells and 18.1 pmol for A_7 cells. Compared with A_7 cells, both potency and maximum inhibition levels of D_2 receptor activation were significantly reduced in M_2 cells.

1 h and treated with M2 anti-FLAG antibody (10 μg/ml; Eastman Kodak, New Haven, CT) in 5% BSA overnight at 4°C. The plates were then washed three times with PBS and treated with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Santa Cruz Biotech) for 1 h. The antibody-labeled cells were then rinsed three times for 5 min each before viewing by confocal microscopy. Confocal microscopy was performed on a Bio-Rad MRC confocal laser microscope equipped with a Nikon Diaphoto 200 inverted microscope using a Nikon 60× 1.40 NA oil-immersion objective. FITC was excited with a 488-nm argon/krypton laser and emitted fluorescence was detected with a 515–540 nm band pass filter.

Statistical Analysis. Statistical analysis was carried out by unpaired Student's *t* test.

Results

Binding of ABP-280 to the Third Cytoplasmic Loop of the Dopamine D_2 Receptor. To identify proteins that bind to the dopamine D_2 receptor, a yeast two-hybrid screen was performed using the entire third cytoplasmic loop of the human D_2 receptor (long form, residues 211–372). A single clone (represented identically in three distinct colonies) encoding part of ABP-280 (residues 1779–2134, known as ABP repeats 16 to 19) was isolated from a human brain cDNA library. The specificity of the D_2-ABP-280 interaction was evaluated by examining the ability of ABP-280 to bind to the entire third cytoplasmic loops of other dopamine receptors. The short form of the D_2 receptor bound to ABP-280 to a similar degree as that of the long form (Table 1). Interestingly, the third cytoplasmic loop of the dopamine D_3 receptor also interacted strongly with ABP-280. As quantified by the activities of β-galactosidase, ABP-280 bound to the D_3 receptor more intensely than the D_2 receptor (Table 1). ABP-280 was found not to interact with the third cytoplasmic loops of either the D_4 receptor, a third member of the D_2 receptor subfamily, or the D_1 receptor, which couples to the stimulation rather than inhibition of adenylate cyclase (Table 1).

The specific interaction of D_2 and D_3 receptors with ABP-280 was further verified by in vitro protein-protein binding. An MBP-ABP280 (residues 1779–2134) fusion protein was purified and incubated with crude GST fusion proteins containing the third cytoplasmic loops of D_2, D_3, D_4, and D_1 receptors. Figure 1 shows that GST-D_2 and GST-D_3 readily bound to MBP-ABP280, whereas no interaction was observed with GST-D_1 and GST-D_4 fusion proteins.

The Association of D_2 Receptors with ABP-280 is Essential for the Efficient Coupling of the Receptor to the Inhibition of Adenylate Cyclase. The availability of human melanoma cell line (M_2) that does not express ABP-280 endogenously allows us to assess D_2 receptor properties in the absence of ABP-280. As a control, the A_7 cell line was generated by stably transfecting M_2 cells with ABP-280 cDNA. Clones stably expressing similar levels of D_2 receptors in M_2 and A_7 cells were selected for further receptor binding and activation studies. Radioligand binding experiments revealed similar affinity for several agonists and antagonists in M_2, A_7, and control D_2 transfected CHO cells (Table 2). Fifty-micromolar GTP treatment reduced the dopamine affinity similarly on both M_2 and A_7 cells, indicating that ABP-280 binding did not seem to affect receptor/G protein interaction directly (Table 2). However, a significant difference was observed in agonist-mediated inhibition of forskolin-stimulated cAMP production. Figure 2 shows that dopamine was less potent in inhibiting forskolin-stimulated adenylate cyclase in M_2 than in A_7 cells (EC_{50} = 33.8 and 3.1 nM, respectively; Table 3). The maximum inhibition of D_2 receptor activation by dopamine was also reduced in M_2 cells (42% compared with 78% in A_7 cells; Fig. 2, Table 3). Similar results were observed with a synthetic D_2 agonist 6,7-ADTN (Fig. 2, Table 3). As a control study, we found that both the potency and maximal inhibition of dopamine in A_7 cells were comparable with those found using a CHO cell line (Fig. 2, Table 3) and to published results using other cell lines (Missale et al., 1998). These findings indicate that the D_2 receptor is less efficient in coupling to the inhibition of adenylate cyclase in the absence of ABP-280.

Regulation of the D_2 Receptor and ABP-280 Interaction by Protein phosphorylation. $GPIb_\alpha$ of the GP Ib-IX complex, the platelet von Willebrand factor receptor that mediates the initial attachment of platelets at a site of injury, has been shown to bind to the same region of ABP-280 as does the D_2 receptor (Meyer et al., 1998). $GPIb_\alpha$ requires a 30-amino acid domain at its carboxyl terminus for ABP association (Andrews and Fox, 1992). This region displays considerable homology with the carboxyl domain of the D_2 and D_3, but not the D_1 or D_4, third cytoplasmic loops (Fig. 3). We tested whether this receptor region is critical for binding to ABP-280. Table 1 shows that elimination of this stretch (D_2Δ314–368) abolished the ability of the D_2 receptor to associate with ABP-280. Within this domain, there exists a conserved serine residue that is a putative PKC phosphorylation site, found in D_2 and D_3 receptors but again not in D_1 and D_4 receptors (Fig. 3). To determine whether this potential phosphorylation site may be involved in the regulation of the ABP-280-D_2 interaction, we replaced this serine residue with aspartic acid (D_2S358D) to mimic its phosphorylated state. Table 1 shows that the D_2S358D mutant receptor

TABLE 3

Inhibition of forskolin-stimulated cAMP production of dopamine D_2 receptors

D_2 receptors were stably expressed in M_2, A_7, and CHO cells. All were stimulated with 10 μM forskolin and increasing concentrations of dopamine or 6,7-ADTN. The EC_{50} values (nM) and the maximum inhibition levels (%) were calculated from the dose-response curves of cAMP production. Data shown are the mean ± S.E. from three to five experiments, indicated in parentheses. D_2 receptor expression levels were 2.48 pmol/mg of protein (M_2) versus 2.55 pmol/mg of protein (A_7). The stable CHO cell clone used contained receptor levels of 1.07 pmol/mg of protein.

	EC_{50} (nM)		Maximum Inhibition (%)	
	Dopamine	6,7-ADTN	Dopamine	6,7-ADTN
A_7-D_2	3.09 ± 0.46 (3)	0.28 ± 0.06 (5)	78.83 ± 2.73 (3)	79.68 ± 4.31 (5)
M_2-D_2	33.78 ± 1.08 (4)*	4.90 ± 0.73 (3)*	42.03 ± 5.19 (4)*	33.79 ± 3.65 (3)*
CHO-D_2	3.17 ± 0.50 (3)	ND	86.70 ± 0.57 (3)	ND

*$P < .05$, compared with A_7-D_2 or CHO-D_2 cells, unpaired Student's *t* test.
ND, not determined.

displayed significantly reduced binding to ABP-280 compared with the wild-type receptor.

To elucidate further the functional significance of serine-358, we stably transfected D_2S358D receptors into A_7 and M_2 cells. Figure 4A shows that the agonist potency of D_2S358D was impaired in A_7 cells, with a reduction also in the maximal inhibition of forskolin-stimulated cAMP production (Fig. 4A, Table 4). In M_2 cells, however, no shifts in agonist potency or maximal signaling between wild-type and D_2S358D receptors were observed, indicating that the effects of this mutation are specific only to ABP-containing cells (Fig. 4B; Table 4). Thus, a structural mimic of serine-358 phosphorylation negatively regulates D_2 receptor association with ABP-280 as well as D_2 receptor signaling in a parallel fashion. Next, we examined the effects of PKC activation on D_2 signaling. Table 4 shows that PMA treatment reduced the agonist potency of D_2 in A_7 but not in M_2 cells. This reduction in potency was similar to that of the D_2S358D phosphorylation mimic observed in A_7 cells. Notably, there was no further shift in agonist potency of the D_2S358D receptor on treatment with PMA (Table 4). Hence, PKC activation can modulate D_2 receptor signaling in a manner consistent with a regulatory role in ABP-280-D_2 association.

ABP-280 Affects the Cell Surface Expression Pattern of the D_2 Receptor. Several intracellular proteins known to

D_2	FEIQTMPNGKTRT**S**LKTMSRRKLS
D_3	LEVRKLSNGRLST**S**LRLGPLQPRG
GPIb$_\alpha$	LLFLRGSLPTFRSSLFLLWVRPNG
D_4	DAVRAAALPPQTPPQTRRRRRAKI
D_1	NCQTTTGNGKPVECSQPESSFKMS

Fig. 3. Alignment of the peptide sequences from the region of GPIb$_\alpha$ that is known to bind to ABP-280, and the C-terminal region of the third cytoplasmic loops of D_1 to D_4 receptors. All the peptides were compared with GPIb$_\alpha$ peptide. Gray boxes depict conserved amino acid residues. A putative PKC phosphorylation site in D_2 and D_3 receptors was indicated in bold.

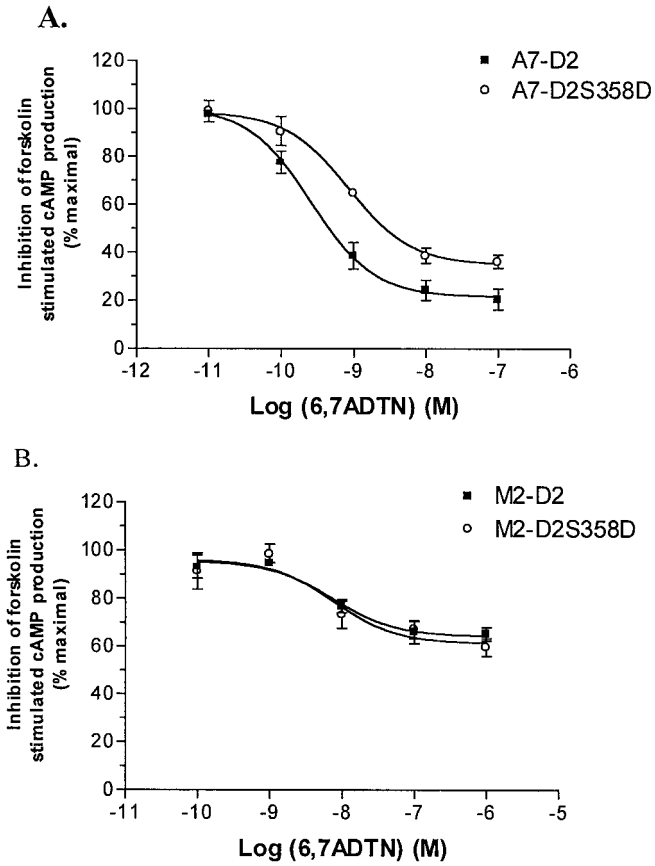

Fig. 4. Dose-response curves of 6,7-ADTN-stimulated inhibition of cAMP accumulation in wild-type (D_2) and mutant (D_2S358D) D_2 receptors. Both receptors were stably transfected into A_7 (A) and M_2 (B) cells. Stable clones with similar receptor expression levels were tested. The expression levels were 1.48 pmol/mg protein (A_7-D_2) versus 1.6 pmol/mg protein (A_7-D_2S358D) and 1.45 pmol/mg protein (M_2-D_2) versus 1.3 pmol/mg protein (M_2-D_2S358D). Dose-response curves were determined as in Fig. 2. Data shown are the mean ± S.E. from three independent experiments.

TABLE 4

The effects of protein phosphorylation on 6,7-ADTN-stimulated D_2 receptor signaling.

D_2 and D_2S358D receptors were stably expressed in M_2 and A_7 cells. The EC_{50} values (nM) and the maximum inhibition (%) were calculated from the dose-response curves of cAMP production. In PMA experiments, cells were incubated with forskolin (10 μM) and increasing concentrations of 6,7-ADTN in the presence of 100 nM PMA (10 min preincubation). PMA treatment alone did not change basal and forskolin-stimulated cAMP production. Stable clones with similar receptor expression levels were tested. The expression levels were 1.48 pmol/mg of protein (A_7-D_2) versus 1.6 pmol/mg of protein (A_7-D_2S358D) and 1.45 pmol/mg of protein (M_2-D_2) versus 1.3 pmol/mg of protein (M_2-D_2S358D). Data shown are the mean ± S.E. from three to four experiments, indicated in parentheses.

	EC_{50} (nM)	Maximum Inhibition (%)
A_7-D_2	0.25 ± 0.10 (4)	79.50 ± 4.29 (4)
A_7-D_2 + PMA	0.83 ± 0.13 (3)*	81.18 ± 0.96 (3)
A_7-D_2S358D	0.75 ± 0.15 (4)*	64.84 ± 2.73 (4)*
A_7-D_2S358D + PMA	1.24 ± 0.37 (3)	72.60 ± 2.44 (3)
M_2-D_2	3.95 ± 0.67 (4)	34.30 ± 4.71 (4)
M_2-D_2 + PMA	4.03 ± 0.98 (3)	40.32 ± 5.20 (3)
M_2-D_2S358D	6.13 ± 0.95 (3)	39.40 ± 3.57 (3)

*$P<.05$, compared with A_7-D_2 cells, unpaired Student"s t test.

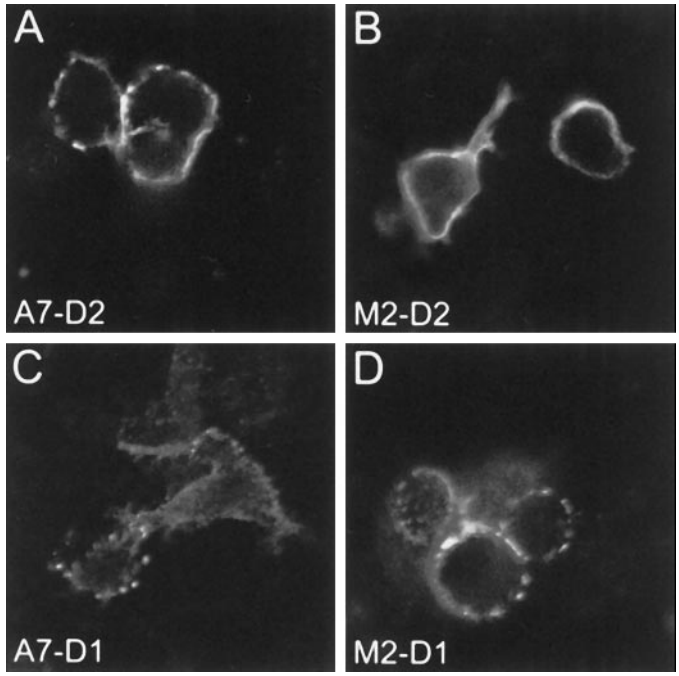

Fig. 5. Plasma membrane expression of FLAG-D_1 and FLAG-D_2 receptors in transiently transfected A_7 and M_2 cells. D_2 receptor expression levels on A_7 and M_2 cells in this specific experiment are 1.2 pmol/mg protein and 1.0 pmol/mg protein, respectively. FLAG-D_2 receptors displayed membrane clustering in A_7 cells (A), but more uniform surface distribution in M_2 cells (B). In contrast, FLAG-D_1 receptors showed a similar clustering appearance in both A_7 (C) and M_2 (D) cells. Representative viewing fields were chosen from three independent experiments.

interact with cell surface receptors have been found to be involved in receptor targeting (Gomperts, 1996). To visualize the cellular distribution of D_2 receptors, we attached a FLAG epitope to the amino-terminus of the receptors (Vickery and von Zastrow, 1999). FLAG-tagged D_2 receptors expressed on A_7 and M_2 cells showed similar affinity to agonist 6,7-ADTN compared with untagged D_2 receptors (K_i = 284 ± 72 and 222 ± 46 nM, respectively; n = 3; Table 2). FLAG-tagged D_2 were then transiently transfected into A_7 and M_2 cells, labeled with FITC-conjugated secondary antibodies and visualized by confocal microscopy. D_1 receptors, also tagged with FLAG at the amino terminus, were used as control receptors. Both D_2 and D_1 receptors displayed clustering in A_7 cells (Fig. 5, A and C). However, in ABP-280-deficient M_2 cells, although D_1 receptors maintained a clustering appearance, D_2 receptors were more uniformly distributed along the plasma membrane (Fig. 5, B and D). These results indicate that ABP-280 contributes to D_2 receptor clustering on the cell surface and may serve to anchor these receptors in prime locations for efficient cellular response to agonist stimulation.

Discussion

In this study, we found that the dopamine D_2 receptor binds to ABP-280, a cytoskeleton-associated protein. Several other neuroreceptors have been reported to associate with cytoskeletal elements. For instance, subtypes of the *N*-methyl-D-aspartate receptor have been shown to bind to α-actinin-2, also a member of the actin-binding protein family, in postsynaptic densities of cortical neurons (Wyszynski et al., 1997). In addition, several ligand-gated ion channels are known to be linked to the cytoskeleton via different adaptor proteins (Kirsch and Betz, 1993; Carbonetto and Lindenbaum, 1995; Wang et al., 1999). Most interestingly, recent reports suggest a similar linkage between GPCRs and the cytoskeleton. For example, the carboxyl terminus of the somatostatin receptor has been shown to associate with the cytoskeleton via cortactin-binding protein (Zitzer et al., 1999). Our data indicate that D_2 receptors can bind directly to ABP-280 with important functional consequences.

The association between the D_2 receptor and ABP-280 was shown to enhance receptor signaling. In M_2 melanoma cells that do not express ABP-280, the ability of D_2 receptors to inhibit forskolin-stimulated adenylate cyclase activity was greatly reduced. We have further shown that mimicking protein phosphorylation by substitution of serine-358 with aspartic acid within the ABP-association domain reduces the binding ability and signal coupling efficiency of the mutant D_2 receptor (Table 1, Table 4). Direct stimulation of PKC with PMA also reduces D_2 signaling (Table 4). This phosphorylation regulation thus suggests a dynamic feature of receptor-cytoskeletal interactions and their potential for regulation by physiological stimuli. PKC activation can occur through multiple pathways. In particular, many GPCRs are capable of signaling homologous and heterologous receptor desensitization through PKC-dependent mechanisms (Chuang et al., 1996). However, it is not clear exactly how PKC-mediated phosphorylation mechanisms alter receptor-signaling pathways. Our findings suggest that one of these mechanisms may involve a regulated association with cytoskeletal components.

It is well known that D_2 autoreceptors are more sensitive to agonists than their postsynaptic counterparts (Skirboll et al., 1979), but the underlying mechanisms for this differential sensitivity remain elusive. The involvement of distinct D_2 receptor protein sequences is unlikely (Skirboll et al., 1979; Clark and White, 1987; Missale et al., 1998). Our results indicate that the differential agonist sensitivity of the presynaptic and postsynaptic D_2 receptors may result from differential neuronal compartmental interactions between D_2 receptors and ABP-280. It would be interesting to examine whether the in vivo interaction between ABP-280 and the D_2 receptor is more prevalent presynaptically than postsynaptically.

The exact mechanism by which ABP-280 modulates the signaling of D_2 receptors is still under investigation, although it is likely that this cytoskeletal component acts as a scaffolding protein. By clustering the components of the signaling pathways together, scaffolding molecules can greatly increase the efficiency of receptor-effector coupling. It has been shown that in *Drosophila melanogaster*, INAD, a protein with five distinct PSD-95/DlgA/ZO-1 (PDZ) domains, serves as a scaffold in the assembly of a highly organized phototransduction pathway that includes receptor, effectors, and regulators, thereby endowing the signaling pathway with extremely high fidelity (Montell, 1998). Our data indicate that ABP-280 assists in the clustering of D_2 receptors to specific cell surface locales. Whether these regions are also enriched in signaling intermediates of D_2 receptors remains to be determined. Studies have demonstrated that the distribution of G proteins in the plasma membrane is not random (Wang et al., 1989) and that G proteins or adenylate cyclase could also be attached to components of the cytoskeleton (Graeser and Neubig 1993; Neubig, 1994). Thus by anchoring receptors as well as signaling molecules, the cytoskeleton may ensure rapid and efficient signal transduction.

ABP-280 is an abundant cytoplasmic protein with an amino terminal actin-binding domain of approximately 275 amino acids, followed by 24 tandem repeats, each approximately 96 amino acids in length (Gorlin et al., 1990). In addition to organizing actin fibers, distinct repeats of ABP-280 have been shown to interact with a number of membrane proteins, including $GPIb_\alpha$, the α subunit of glycoprotein Ib (Andrews and Fox, 1992; Meyer et al., 1998), integrin (Sharma et al., 1995), furin (Liu et al., 1997), as well as the cytosolic stress-activated protein kinase kinase (Marti et al., 1997). Interestingly, these interactions occur in distinct ABP repeat domains with unique functional consequences, ranging from effects on cytoskeletal organization to the inhibition of receptor internalization and effector coupling. Notably, the carboxyl-terminal repeat (number 24) of ABP-280 contains a self-assembly sequence that forms homodimers. Conceivably, these structural features and multiple interaction domains of ABP-280 may enable the formation of receptor-effector complexes necessary for the efficient signaling of D_2 receptors and their proper membrane targeting, as our results suggest.

Recently, the third cytoplasmic loop of D_2 receptors was shown to interact with another protein, spinophilin (Smith et al., 1999). Although the functional significance of this association is still unknown, it has been suggested that, like ABP-280, spinophilin may play a role as a scaffolding protein in organizing the D_2 receptor-signaling complex. Spinophilin is also a cytoskeletal-associated protein; it contains an actin-

binding domain (Satoh et al., 1998). In addition, spinophilin has a carboxyl-terminal coiled-coil structure and a single consensus PDZ domain. It has been shown that spinophilin interacts with protein phosphatase-1 at a site distinct from the D_2 binding site. Hence, the multiplicity of G protein-independent interactions with the D_2 receptor seem to enable a diversity of functional regulatory processes, ranging from the inhibition of adenylate cyclase, as we have shown, to the potential phosphatase-mediated activity against competing stimulatory kinase pathways.

In conclusion, we have identified a novel association between the D_2 receptor third cytoplasmic loop and the actin cytoskeleton via ABP-280. This association enhances receptor signaling and can be regulated by protein phosphorylation. Further studies to establish this association in neuronal cells will be of great value in understanding the role of the cytoskeleton in dopaminergic neurotransmission.

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