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Novel Interaction of the Dopamine D_2 Receptor and the Ca^{2+} Binding Protein S100B: Role in D_2 Receptor Function

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ABSTRACT

S100B is a calcium-binding protein with both extracellular and intracellular regulatory activities in the mammalian brain. We have identified a novel interaction between S100B and the dopamine D_2 receptor. Our results also suggest that the binding of S100B to the dopamine D_2 receptor enhances receptor signaling. This conclusion is based on the following observations: 1) S100B and the third cytoplasmic loop of the dopamine D_2 receptor interact in a bacterial two-hybrid system and in a poly-histidine pull-down assay; 2) immunoprecipitation of the D_2 receptor also precipitates FLAG-S100B from human embryonic kidney 293 cell homogenates and endogenous S100B from rat neostriatal homogenates; 3) S100B immunoreactivity was detected in cultured neostriatal neurons expressing the D_2 receptor; 4) a putative S100B binding motif is located at residues 233 to 240 of the D_2 receptor, toward the amino terminus of the third cytoplasmic loop. D_3-IC3, which does not bind S100B, does not contain this motif; and 5) coexpression of S100B in D_2 receptor-expressing 293 cells selectively increased D_2 receptor stimulation of extracellular signal-regulated kinases and inhibition of adenylate cyclase.

Interest in dopamine receptor research has been fueled by studies of brain diseases such as Parkinson's disease and schizophrenia, showing that dopamine has a role in either the pathogenesis or symptoms of the diseases and that substances acting at the receptors act as therapeutic agents (Strange, 1992). The dopamine receptor family is composed of D_1-like (D_1 and D_5) and D_2-like (D_{2L}, D_{2S}, D_3, and D_4) receptors (Neve and Neve, 1997). The dopamine D_2 receptor belongs to a subfamily of seven-transmembrane domain G protein-coupled receptors that interact with the G proteins $G\alpha_i$ and $G\alpha_o$ to modulate several effectors, including adenylate cyclase, potassium channels, and mitogen-activated protein kinases (Neve et al., 2004).

Protein-protein interactions are central to most important cellular processes, including DNA replication, transcription, translation, cell cycle control, and signal transduction. The yeast two-hybrid assay is a powerful method for identifying and characterizing protein-protein interactions (Fields and Song, 1989), but it is a tedious procedure, limited by the basic biology of the yeast. Yeast grows slowly, is difficult to transform efficiently, and requires unique reagents and techniques. The bacterial two-hybrid (B2H) system has the following advantages: fast growth rate, high transformation efficiency, and manipulations that are routine in most molecular biology laboratories (Joung et al., 2000). The purpose of this study was to use the B2H system to identify additional proteins that bind to and regulate the function of the D_2 receptor.

S100 proteins comprise an extremely diverse and highly specialized family of approximately 21 Ca^{2+}-binding proteins (Donato, 1999; Zimmer et al., 2003; Marenholz et al., 2004). An S100 protein is typically a low molecular mass protein (molecular mass between 9 and 13 kDa) characterized by the presence of two Ca^{2+}-binding sites of the EF-hand type (Donato, 2003). S100 proteins have been implicated in the regulation of protein phosphorylation, Ca^{2+} homeostasis, enzyme activity, gene transcription, cell growth and differentiation, and the inflammatory response (Schäfer and Heizmann, 1996; Donato, 1999). Alterations of S100 function have

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ABBREVIATIONS: B2H, Bacteriomatch Two-Hybrid; 3-AT, 3-amino-1,2,4-triazole; CaM, calmodulin; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; G protein, heterotrimeric GTP-binding protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEK, human embryonic kidney; D_2-IC3, the third intracellular loop of the D_2 receptor; MAP2, microtubule-associated protein-2; PTX, pertussis toxin; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; coIP, coimmunoprecipitation; PCR, polymerase chain reaction; 7-OH DPAT, 7-hydroxy-2-dipropylaminotetralin.

been implicated in many diseases, including cancer, Down's syndrome, Alzheimer's disease, cardiomyopathy, psoriasis, cystic fibrosis, amyotrophic lateral sclerosis, and epilepsy (Heizmann, 2002; Heizmann et al., 2002). Thus, S100 proteins may be important diagnostic markers and therapeutic targets. The results of clinical studies on the S100 protein S100B in schizophrenia suggest that patients suffering from schizophrenia have increased S100B serum concentrations in the acutely psychotic stage of disease (Rothermundt et al., 2004). S100B is an acidic protein with a molecular mass of 21 kDa as a homodimer, and it is perhaps the best characterized of the S100 proteins (McClintock and Shaw, 2000). S100B has no known enzymatic function and exerts its intracellular effects by interacting with and modulating the activity of other proteins. In vitro, S100B interacts with more than 20 substrates in a Ca^{2+}-sensitive manner (Donato, 1999).

We now describe a novel interaction between S100B and the dopamine D_2 receptor, identified using the B2H system. We confirmed the novel interaction using coimmunoprecipitation in human embryonic kidney (HEK) 293 cells and in rat neostriatum. We identified S100B immunoreactivity in D_2 receptor-expressing neostriatal neurons. We determined that the third intracellular loop of the D_2 receptor (D_2-IC3) is a contact point for the interaction with S100B by a histidine-tagged pull-down assay. S100B bound to IC3 of both D_{2L} and D_{2S} but not D_3. We also proposed a putative binding motif for the interaction by sequence alignment. Finally, we found that coexpression of the D_2 receptor and S100B significantly increased D_2 receptor stimulation of extracellular signal-regulated kinases (ERKs) and inhibition of adenylate cyclase in HEK293 cells.

Materials and Methods

Materials. The B2H System and BacterioMatch II Rat Brain Library were purchase from Stratagene (La Jolla, CA). Quinpirole, 7-OH DPAT, (+)-butaclamol, 3-isobutyl-1-methylxanthine, adenine HCl, *n*-dodecylmaltoside, ethylene-bis(oxyethylenenitrilo)tetraacetic acid, 3-amino-1,2,4-triazole (3-AT), and culture media were purchased from Sigma-Aldrich (St. Louis, MO). Histidine dropout supplement (mixtures of amino acids and other nutrients) was purchased from BD Biosciences Clontech (Palo Alto, CA). [^{3}H]Spiperone (95 Ci/mmol) was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). Fetal and calf bovine sera for cell culture were purchased from HyClone (Logan, UT). The Lipofectamine 2000 cell transfection kit was purchased from Invitrogen (Carlsbad, CA). Precast gels and rat neostriatal neurons were purchased from Lonza Walkersville (Walkersville, MD). Protein G Plus agarose, normal rabbit IgG, and normal mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies used include: rabbit anti-dopamine $D_{2L/S}$ (1/500 dilution; Millipore, Billerica, MA), mouse anti-S100B (1/1000 dilution; GeneTex, San Antonio, TX), rabbit anti-His (1/500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-FLAG M2 (1/1000 dilution; Sigma), rabbit anti-λ-cI antibody (1/1000 dilution; Stratagene), rabbit anti-myc (1/1000 dilution; Bethyl, Montgomery, TX), mouse anti-myc (1/1000 dilution; Millipore), rabbit anti-dually phosphorylated (i.e., activated) ERKs (1/1000 dilution; Invitrogen), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1/50,000 dilution; Millipore), and rabbit anti-microtubule-associated protein-2 (MAP2) (1/1000 dilution; Abcam, Cambridge, MA). Alexa Fluor 568-labeled goat anti-mouse IgG antibody, Alexa Fluor-486 goat anti-rabbit IgG antibody, and Prolong anti-fade kit were obtained from Invitrogen. S100B was from US Biological (Swampscott, MA). The cAMP enzyme immunoassay kit was from Cayman Chemical (Ann Arbor, MI). The BCA protein assay kit, secondary antibodies for immunoblot analysis, and the Super-Signal West Pico chemiluminescent kit were from Pierce Biotechnology (Rockford, IL). Protease inhibitor cocktail (set III) was from EMD Biosciences (San Diego, CA). HEK293 cells, a transformed cell line from human embryonic kidney, were purchased from American Type Culture Collection (Manassas, VA).

DNA Constructs for Bacterial Two-Hybrid Assay. The sequence encoding D_2-IC3, amino acids 206 to 375 (leucine to methionine), was amplified by PCR and subcloned in-frame with the λ-cI DNA-binding domain into pBT (B2H System; Stratagene) to generate pBT-D_2-IC3 as "bait." The construct was verified by DNA sequencing, and the presence of λ-cI-tagged D_2-IC3 with the expected molecular size was also verified by immunoblot using anti-λ-cI antibody. A B2H rat brain cDNA library (as a "target") was purchased from Stratagene. It contains pooled rat brain tissues (Sprague-Dawley, male, 10 weeks). The vector is pTRG, and the average insert size is approximately 1.8 kilobases.

Bacterial Two-Hybrid Screening. The B2H System reporter strain competent cells (Stratagene) were transformed with pBT-D_2-IC3 and the BacterioMatch II Rat Brain Library (Stratagene) according to the manufacture's protocol (Stratagene). Detection of protein-protein interaction is based on transcriptional activation of the *HIS3* reporter gene, which allows growth in the presence of 3-AT (5–20 mM), a competitive inhibitor of the His3 enzyme. Positives are verified using the *aadA* gene, which confers streptomycin resistance, as a secondary reporter. All positive clones were analyzed by DNA sequencing. To validate the putative protein-protein interactions, we then retransformed the reporter strain with the isolated target plasmid plus bait plasmid as described by the manufacturer (Stratagene).

Cell Culture, Transfection, and Selection. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% calf bovine serum, penicillin-streptomycin, appropriate selection antibiotics (G418 sulfate 600 μg/ml; puromycin 2 μg/ml), and grown in a humidified incubator at 37°C in the presence of 10% CO_2.

The creation of a cell line stably expressing a c-myc-tagged D_{2L} dopamine receptor (referred to hereinafter as the D_2 receptor) was described in a previous report (Liu et al., 2007). Because HEK293 cells have endogenously expressed S100B detectable via immunoblotting using anti-S100B antibody, a cell line stably coexpressing c-myc-tagged D_2 dopamine receptor and FLAG-tagged S100B (myc-D_2/FLAG-S100B-HEK293) was generated as follows: cDNA encoding rat brain S100B was amplified using the polymerase chain reaction, digested with EcoRI-BamHI, and subcloned into the pcDNA-DNA3 expression vector, placing the FLAG-tag at the NH_2 terminus of S100B. The FLAG-S100B construct was transfected into myc-D_2-HEK cells using Lipofectamine 2000 transfection reagent and selection with puromycin (2 μg/ml) and G418 (600 μg/ml). Cell lines expressing the myc-tagged D_2 receptor and FLAG-tagged S100B were isolated by screening via radioligand binding using [^{3}H]spiperone and via immunoblot analysis using a mouse anti-myc and a mouse anti-FLAG antibody. The binding of [^{3}H]spiperone was assessed as described previously (Liu et al., 2006), and the c-myc-tagged D_2 receptor with coexpression of FLAG-tagged S100B had similar affinity for [^{3}H]spiperone as reported previously for the c-myc-tagged D_2 receptor (Liu et al., 2007). The molar ratio of D_2 to S100B in the myc-D_2/FLAG-S100B-HEK293 cell line was approximately 1:0.9 (data not shown).

Neostriatal Neuronal Cultures. Rat striatal neurons were cultured as follows: the cells were removed from liquid nitrogen and placed in a 37°C water bath for 2 to 3 min and then gently into a 15-ml centrifuge tube, to which was added prewarmed Primary Neuron Growth Medium (Lonza Walkersville) drop-wise into the cells while rotating the tube by hand. The cell suspension was mixed by inverting the tube twice. Cells were plated on 18-mm diameter poly(D-lysine)-coated glass coverslips at a density of 75,000 cells per coverslip and placed in a humidified 5% CO_2

incubator at 37°C. After 4 h, the medium was replaced with fresh, prewarmed medium. After 4 days, the medium was changed, and cells were ready for use after 6 to 8 days in culture. Just fewer than 90% of the cells in the cultures were neurons, as determined by the presence of MAP2 immunoreactivity.

Coimmunoprecipitation of the myc-Tagged D_2 Receptor and S100B, and Endogenous D_2 Receptor and S100B. myc-D_2/FLAG-S100B-HEK293 cells from confluent 10-cm^2 plates were washed and incubated twice for 3 min each time with calcium- and magnesium-free phosphate-buffered saline (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 68 mM NaCl, pH 7.4). Cells were released from the plate using trypsin, triturated, and centrifuged at 600*g*. The cells were resuspended in phosphate-buffered saline with 1.0% *n*-dodecylmaltoside and protease inhibitor cocktail and solubilized on ice for 2 h with gentle shaking. Sprague-Dawley rats (12 weeks old, female) were killed by decapitation, and the heads of the animals were immediately immersed in liquid nitrogen for 6 s. The brains were then removed, and the striatum was rapidly (20 s) dissected out on an ice-cold surface. The tissue was then triturated using fire-polished Pasteur pipettes in phosphate-buffered saline with 1.0% *n*-dodecylmaltoside and protease inhibitor cocktail and solubilized on ice for 8 h with gentle shaking. The insoluble material from the two tissue preparations was removed by centrifugation at 25,000*g* for 30 min. The protein concentration of the supernatant was analyzed by BCA protein assay reagent. An aliquot with 1.5 mg of protein was incubated with 2 μg of rabbit anti-myc antibody (for coIP with myc-D_2/FLAG-S100B-HEK293 cells) or rabbit anti-D_2 antibody (for coIP with neostriatal lysates) at 4°C for 2 h and further incubated with 20 μl of a 50% slurry of Protein G Plus beads overnight at 4°C. Beads were washed, and samples were eluted according to the manufacturer's instructions, separated by SDS-PAGE, and immunoblotted using mouse anti-FLAG antibody for myc-D_2/FLAG-S100B-HEK293 cells or using mouse anti-S100B antibody for endogenous D_2 receptor and S100B.

In Vitro Histidine-Tagged Dopamine Receptor-IC3 Pull-Down Assay. For the construction of the histidine-tagged fusion proteins, D_{2S}-IC3 (amino acids 206 to 346), D_{2L} receptor (D_{2L}-IC3; amino acids 206 to 375), and D_3 receptor (D_3-IC3; amino acids 206–376) were PCR-amplified. The PCR products were cut as BamHI-SalI fragments and subcloned into pET-24a (+) (Novagen, Madison, WI) and then transformed into BL21(DE3)-competent cells (Novagen). Transformants were screened by induction with 0.5 mM isopropyl β-D-thiogalactoside and immunoblot analysis using a rabbit anti-His antibody. For larger-scale purification, the His-tagged dopamine receptor-IC3 clones were grown in Luria broth containing kanamycin (50 μg/ml) at 37°C to A_{600} = 0.5 and induced with 0.5 mM isopropyl β-D-thiogalactoside for 4 h at 23°C. Bacteria were pelleted and washed with phosphate-buffered saline. Pellets were resuspended in B-PER II bacterial protein extraction reagent (Pierce Biotechnology) with 0.5 mg/ml lysozyme (Fermentas, Hanover, MD) and protease inhibitor and incubated for 20 min with gentle rotation at room temperature. The bacterial cell lysates containing the same amount of His-tagged dopamine receptor-IC3 fusion proteins or a hexa-His peptide without insert as control were clarified by centrifugation, and the supernatants were applied to nickel-nitrilotriacetic acid agarose (QIAGEN, Valencia, CA). Prebound, washed beads were incubated with 500 ng of purified S100B overnight at 4°C, followed by wash and elution steps. The eluates were separated by SDS-PAGE, and bound proteins were analyzed by immunoblotting with rabbit anti-S100B antibody.

Confocal Immunofluorescence Imaging. Neostriatal neurons grown on glass coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 68 mM NaCl, pH 7.4) for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, and then blocked with 5% goat serum for 1 h at room temperature. Neurons were incubated with rabbit anti-MAP2 or rabbit anti-$D_{2L/S}$ and mouse anti-S100B at 4°C overnight and then incubated for 1 h with Alexa Fluor 486 goat anti-rabbit IgG (1/1000) and Alexa Fluor-568-tagged goat anti-mouse IgG (1/1000) and followed by five 10-min washes with phosphate-buffered saline. The coverslips were then mounted onto a slide with the ProLong antifade kit, dried in the dark, and scanned alternating between 486 and 568 nm using a Leica TCS SP confocal laser scanning microscope (Leica, Wetzlar, Germany). System settings were held constant for all imaging.

Immunoblotting. Proteins were separated by SDS-PAGE through a 4 to 20% or 10% polyacrylamide gel and transferred to polyvinyl membranes (Millipore). The membranes were blocked for 1 h at room temperature with 5% nonfat milk with 0.05% Tween 20 in Tris-buffered saline (TBS), pH 7.4, at 4°C, washed twice for 5 min, followed by two 10-min washes with TBS, and incubated with primary antibody at room temperature for 2 h or overnight at 4°C. The membranes were washed twice for 5 min, followed by two 10-min washes with TBS, and then incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG or anti rabbit IgG) at room temperature for 1 h. In addition to measuring protein concentrations to ensure equal loading, in many experiments in which phosphorylated forms of ERKs were detected, the membranes were then stripped with Restore Western blot-stripping buffer (Pierce) for 20 min at room temperature, followed by two 5-min washes with TBS, blocked for 1 h at room temperature with 5% nonfat milk in TBS, and then incubated with mouse anti-GAPDH antibody, a housekeeping gene product, to further ensure that equal amounts of proteins were loaded, followed by goat anti-mouse IgG. Immunodetection was accomplished using a SuperSignal West Pico chemiluminescent kit. The intensity of bands was quantified using Gel Doc EQ System (Bio-Rad Laboratories, Hercules, CA). A one-way analysis of variance and Bonferroni post hoc comparison was used to analyze data.

Cell Stimulation for Immunodetection of ERKs. Cells expressing myc-D_2 or myc-D_2/FLAG-S100B were grown in 12-well plates to 80 to 85% confluence. The cells were starved in serum-free Dulbecco's modified Eagle's medium overnight and then incubated with the D_2-like receptor agonist quinpirole or epidermal growth factor at the indicated concentrations for 5 min at 37°C. Incubation was terminated by placing the tissue culture cluster on ice and rapidly aspirating the medium, followed by the addition of ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM $NaVO_3$, protease inhibitors, and phosphatase inhibitor) and incubation for 20 min with shaking. After centrifugation (14,000*g* at 4°C for 15 min), the supernatant was collected, and the protein concentration was measured and adjusted using radioimmunoprecipitation assay buffer. The cell lysates (20 μl) with equal amounts of protein mixed with Laemmli loading buffer were denatured at 70°C for 10 min and separated by SDS-PAGE for immunodetection as described.

cAMP Accumulation Assay. The ability of the D_2 receptor agonist 7-OH-DPAT to inhibit 30 μM forskolin-stimulated cAMP accumulation was measured in intact myc-D_2-HEK293 cells and myc-D_2/FLAG-S100B-HEK293 cells. Cells were plated between 100,000 and 150,000 cells/well in 48-well tissue culture plates and used in experiments 2 to 3 days later. Before the assay, cells were preincubated with Earle's balanced salt solution with 0.2% ascorbic acid, 500 μM 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor), and 2% fetal bovine serum, pH 7.4, for 20 min at 37°C. The cells were placed on ice for the addition of 7-OH DPAT and 30 μM forskolin and then incubated at 37°C. The assay was terminated after 20 min by decanting the medium, and the cells were lysed with 100 μl of 3% trichloroacetic acid. Lysates were stored at 4°C at least 2 h before quantification of cAMP. The amount of cAMP in each well was measured using a cAMP enzyme immunoassay kit (Cayman Chemical).

Results

Identification of D_2 Receptor-Binding Proteins by Bacterial Two-Hybrid Library Screening. A cDNA library screening based on the B2H system was used to identify proteins that bind to the D_2 receptor. For this purpose, the rat

D_{2L}-IC3 coding sequence was subcloned into plasmid pBT (pBT-D_2-IC3) to serve as bait with which to screen a rat brain cDNA library in the pTRG plasmid. In screens of approximately 2.5 million clones, we identified 200 positive clones. To validate the detected protein-protein interactions, bacteria were retransformed with each cloned cDNA and pBT-D_2-IC3. Approximately 40 of the 200 clones reproducibly grew on selective screening medium (3-AT) when cotransformed with the bait protein but failed to grow on selective screening medium when cotransformed with the empty pBT vectors, and thus were verified positives. The DNA sequence analysis revealed that clone number 28 matched the full-length sequence encoding rat S100B (GenBank accession number NM_01319).

Interaction between Dopamine Receptor-IC3 and S100B in Vitro. The in vitro binding of S100B to the third cytoplasmic loops of D_{2S}, D_{2L}, and D_3 was studied using a histidine-tagged dopamine receptor-IC3 pull-down assay. D_{2S}, D_{2L}, and D_3-IC3-His fusion proteins were synthesized in bacteria, purified, immobilized on nickel-nitrilotriacetic acid beads, and incubated with purified recombinant S100B. The bound proteins eluted from the beads were separated by SDS-PAGE. S100B immunoreactivity was detected in the eluates from bacterial cell lysate expressing histidine-tagged D_{2S}-IC3 and histidine-tagged D_{2L}-IC3, but not histidine-tagged D_3-IC3 or hexa-His peptide without insert, confirming that S100B binds to the third intracellular loop of both D_{2S} and D_{2L}, but not D_3 (Fig. 1).

Coimmunoprecipitation of the myc-Tagged D_2 Receptor and FLAG-S100B, and Endogenous D_2 Receptor and S100B. To confirm a direct interaction between full-length D_2 receptor and S100B, we expressed both FLAG epitope-tagged S100B and c-myc-D_{2L} receptor in HEK293 cells. Immunoprecipitation of the D_2 receptor with anti-myc resulted in the precipitation of FLAG-S100B, as indicated by immunoblotting with anti-FLAG antibody (Fig. 2A). FLAG-S100B immunoreactivity was not detected in control cells in which the immunoprecipitation was performed with an irrel-

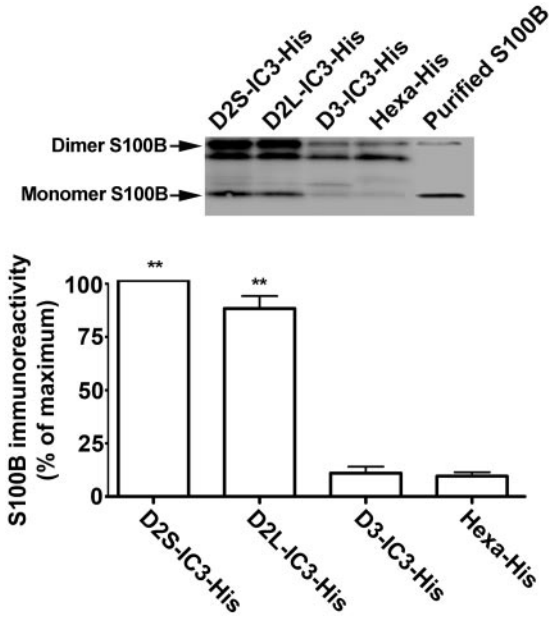

Fig. 1. S100B binds to D_2-IC3 in an in vitro pull-down assay. S100B immunoreactivity was present in the eluates from bacterial cell lysate expressing histidine-tagged D_{2S}-IC3 and histidine-tagged D_{2L}-IC3 but not histidine-tagged D_3-IC3 or hexa-His peptide without insert, demonstrating a specific interaction between the third cytoplasmic loop of the dopamine D_2 receptor and S100B. The construction of histidine-tagged receptor fragments and the in vitro pull down assay protocol were as described under *Materials and Methods*. Top, lanes D_{2S}-IC3-His, D_{2L}-IC3-His, and D_3-IC3-His show the result of a representative pull-down assay, and lane Hexa-His is the no-insert control for that experiment. The right lane is a positive control for immunoblotting (50 ng of purified S100B). Bottom, mean ± S.E. from three independent experiments. **, $p < 0.01$ compared with the no-insert control, paired t test

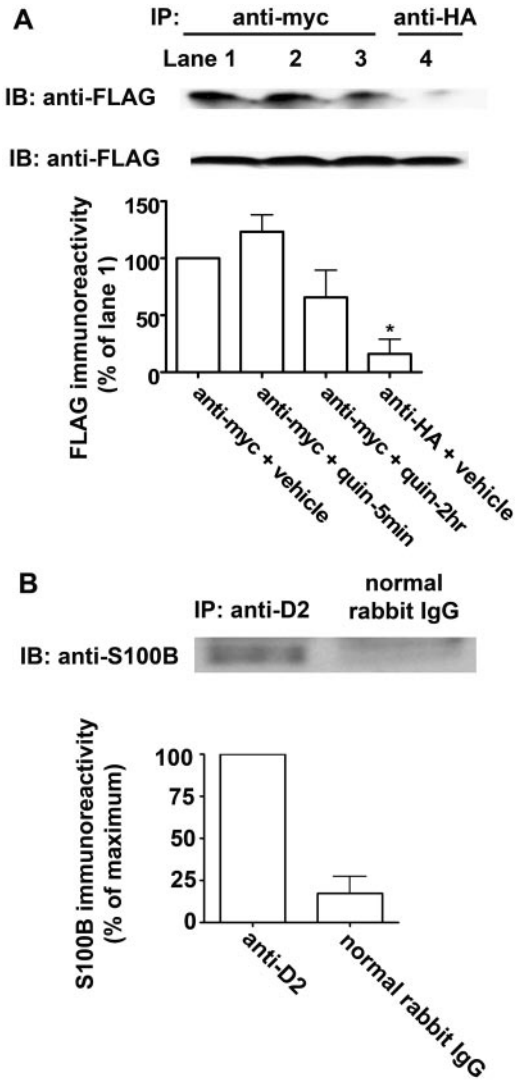

Fig. 2. Coimmunoprecipitation of dopamine D_2 receptor and S100B. A, coimmunoprecipitation from HEK293 cells. Top, representative coIP of myc-D_2 and FLAG-S100B from myc-D_2/FLAG-S100B-HEK293 cells is shown. FLAG immunoreactivity was present in the eluates when the immunoprecipitation was with anti-myc (lanes 1–3) but not when using an irrelevant antibody to a hemagglutinin epitope (anti-HA, lane 4). In addition, FLAG-S100B immunoreactivity was similar in eluates from cells treated with vehicle (lane 1) or with the D_2 receptor agonist quinpirole (1 μM) for 5 min or 2 h (lanes 2 and 3). Middle, immunoblot analysis of the input material with anti-FLAG suggests that all immunoprecipitation samples had similar amounts of FLAG-S100B. Bottom, the results shown are the mean ± S.E. from three independent experiments. There was no significant difference between samples prepared from vehicle- or quinpirole- (quin) treated cells ($p > 0.05$). B, coimmunoprecipitation from rat striatal homogenate. Top, S100B immunoreactivity was present in the eluates when anti-D_2 was used to precipitate the D_2 receptor (lane 1), with little present when normal rabbit IgG was used (lane 2). Bottom, results shown are the mean ± S.E. from three independent experiments, demonstrating a constitutive interaction between the endogenous dopamine D_2 receptor and S100B in rat neostriatum. *, $p < 0.05$ compared with anti-myc + vehicle, Bonferroni post hoc comparison.

evant antibody to a hemagglutinin epitope. The amount of S100B immunoreactivity in eluates from cells treated with the D_2-like receptor agonist quinpirole (10 μM) for 5 min or 2 h was similar to that of cells treated with vehicle. Immunoprecipitation of the endogenous D_2 receptor from rat neostriatum also resulted in the precipitation of S100B immunoreactivity; S100B immunoreactivity was not detected in control samples in which the immunoprecipitation was performed with rabbit normal IgG (Fig. 2B). Therefore, the D_2 dopamine receptor specifically and constitutively bound S100B in HEK293 cells and in rat neostriatum.

Detection of Endogenous S100B in Primary Neostriatal Neurons. S100B is expressed most abundantly in astroglial cells (Suzuki et al., 1987). To determine whether S100B is also expressed in neostriatal neurons, which express the D_2 receptor, the colocalization of immunoreactivity for S100B and the neuronal marker MAP2 (Fig. 3, A–C) and for S100B and D_2 receptor (Fig. 3, D–F) was determined in neostriatal neuronal cultures. In 12 visual fields from 3 independently prepared cultures, all cells that expressed immunoreactivity for MAP2 (green; 85 neurons) also expressed S100B (red). In two additional experiments, we assessed the colocalization of immunoreactivity for S100B and the D_2 receptor. In 8 visual fields with 83 cells exhibiting a neuronal morphology, ~75% of the cells expressed both S100B (red) and the D_2 receptor (green) (Fig. 3).

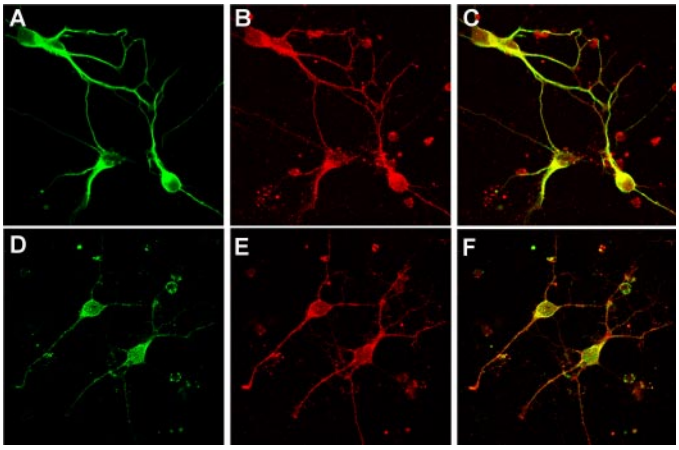

Fig. 3. Confocal imaging of S100B. Top row, representative confocal fluorescence images depict immunoreactivity for MAP2 (A) and S100B (B) in neostriatal neurons. In the merged image (C), pixels containing immunoreactivity for both S100B and the neuronal marker MAP2 appear as yellow. Bottom row, representative confocal fluorescence images depict immunoreactivity for D2 (D) and S100B (E) in neostriatal neurons. In the merged image (F), pixels containing immunoreactivity for both S100B and the D_2 receptor appear as yellow.

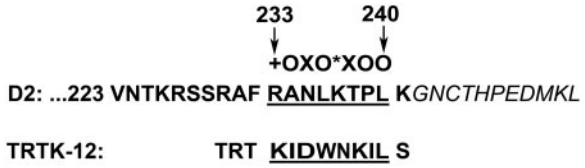

Fig. 4. A putative S100B-binding motif is located near the N terminus of the third intracellular loop of the D_2 receptor. The amino acid sequence for the rat D_2 receptor was analyzed based on the previous reports (Ivanenkov et al., 1995; Bianchi et al., 1996; McClintock and Shaw, 2000). A putative S100B binding motif close to the N terminus (amino acids: 233–240) of the third intracellular loop of the D_2 receptor is aligned with TRTK-12 peptide characterized by Ivanenkov et al. (1995). (+, basic residue; O, hydrophobic residue; *, hydrophilic residue; X, any residue). Italics denote residues at the beginning of the alternatively spliced region that distinguishes D_{2L} from D_{2S}.

Putative S100B Binding Motif. The identification of an S100B binding "epitope" from bacteriophage studies provides a useful probe to search for a binding motif. Ivanenkov et al. (1995) screened a bacteriophage random peptide display library and identified the consensus sequence +OXO*XOO (+, basic; O, hydrophobic; *, hydrophilic; X, variable) as the "epitope" binding region for S100B. The peptide TRTKIDWNKILS (TRTK-12), a 12-residue peptide containing the consensus sequence, successfully competes with other S100B binding proteins such as glial fibrillary acidic protein and

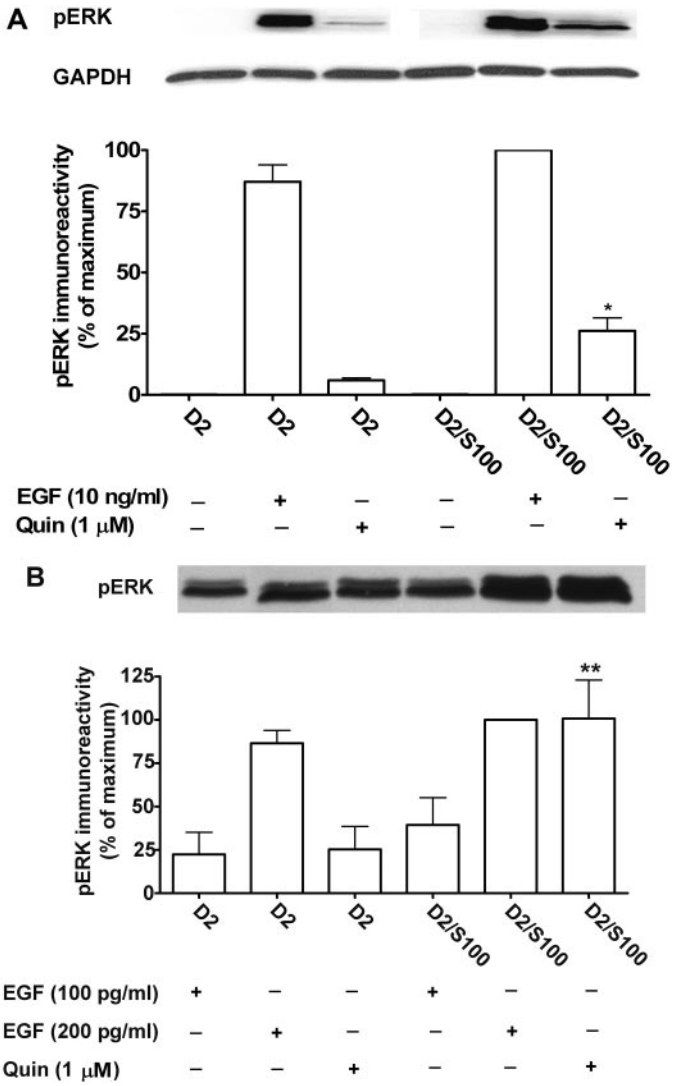

Fig. 5. Coexpression of S100B with the D_2 receptor in HEK293 cells modulates receptor activation of ERKs. A, compared with the nontreated samples, treatment with 1 μM concentration of the D_2 receptor agonist quinpirole (Quin) at 37°C for 5 min induced rapid and robust activation of ERKs in HEK293 cells expressing the D_2 receptor (D2), and stimulation was increased in cell lines expressing both D_2 and S100B (D2/S100). Top, a representative immunoblot for activated ERK (pERK) and GAPDH as a loading control. The results shown in the bottom are the mean ± S.E. from three (EGF) or four (Quin) independent experiments. B, top, a representative immunoblot for activated ERK (pERK) in cells expressing the D_2 receptor alone (D2) or with S100B (D2/S100B) and treated with 1 μM quinpirole (Quin) or EGF at 100 or 200 pg/ml. There was no significant difference between the two cell lines for any concentration of EGF ($p > 0.05$). *, $p < 0.05$; **, $p < 0.01$ compared with the same treatment in the cell line expressing D_2 alone, Bonferroni post hoc comparison.

CapZ for calcium-sensitive S100B binding (Ivanenkov et al., 1995; Bianchi et al., 1996). This consensus sequence for S100B binding has been identified in 25 proteins found to interact in vitro with S100B (McClintock and Shaw, 2000). We scanned the D_2 receptor for this motif and identified a potential S100B binding site at amino acid residues 233 to 240 close to the amino terminus of the third intracellular loop and adjacent to the alternatively spliced region of the receptor (Fig. 4). We did not find this motif in the third intracellular loop of the D_3 receptor or in the D_4 receptor.

Expression of S100B with the Dopamine D_2 Receptor Modulates Receptor Activation of ERKs. Receptor-stimulated activation of ERKs (ERK1, 44 kDa; ERK2, 42 kDa) was measured using an antibody for phospho-ERKs to quantify the abundance of dually phosphorylated ERKs. HEK293 cells expressing D_2 receptor alone (myc-D_{2L}-HEK293) or expressing D_2 receptor and FLAG-S100B (myc-D_{2L}/FLAG-S100B-HEK293) were selected to express similar D_2 receptor densities (B_{max} values for myc-D_{2L}-HEK293 and myc-D_{2L}/FLAG-S100B-HEK293 were 4660 ± 230 and 4250 ± 700 fmol/mg of protein, respectively). Treatment with the D_2 receptor agonist quinpirole (1 μM) induced rapid and robust activation of ERKs in HEK293 cells expressing the myc-D_2 receptor (Fig. 5A). The stimulation of ERKs by quinpirole was significantly increased in cells coexpressing FLAG-S100B.

To determine whether S100B enhanced activation of ERKs at a step downstream from the D_2 receptor, we assessed the effect of S100B expression on epidermal growth factor (EGF)-induced activation of ERKs. Coexpression of S100B had no significant effect on the ERK response to EGF (10 ng/ml) (Fig. 5A). To confirm that the lack of effect of S100B on the response to EGF was not because ERK was already maximally activated, we also tested lower concentrations of EGF (100 and 200 pg/ml). Although there was a tendency for EGF-induced activation of ERK to be enhanced in cells expressing S100B, the effect was not statistically significant, in contrast to the robust enhancement of quinpirole-induced activation of ERK in cells coexpressing S100B (Fig. 5B).

To verify that EGF-induced signaling was independent of D_2 receptor activation of $G\alpha_{i/o}$, we used pertussis toxin (PTX) to inactivate this class of G proteins. Treatment of HEK293 cells with PTX (50 ng/ml overnight) abolished D_2 receptor activation of ERKs without altering EGF-induced activation (Fig. 6). Overall, these results imply that the increased ERK activation with coexpression of S100B is primarily due to the interaction of D_2 and S100B rather than to binding of S100B to downstream elements that are shared between signaling pathways of the EGF and D_2 receptors.

Increased Inhibition of Forskolin-Stimulated cAMP Accumulation in Cells Expressing S100B. We also characterized the effect of coexpression of S100B on D_{2L} receptor inhibition of adenylate cyclase. The experiment shown in Fig. 7, representative of three independent experiments, demonstrates that the potency of the agonist is unchanged by the coexpression of S100B, but maximal inhibition of forskolin-stimulated cAMP accumulation is significantly increased. The average EC_{50} values for the D_2 and the D_2/S100B cell lines were 21 and 15 nM, respectively. The maximal inhibition of forskolin-stimulated cAMP accumulation for the D_2 and D_2/S100B cell lines was 57 ± 12% and 66 ± 9%, respectively ($p < 0.05$, by paired t test, $n = 3$), respectively. This is qualitatively similar to the effect of S100B coexpression on quinpirole-induced activation of ERK.

Discussion

Dopamine receptors are important molecules underlying neuropsychiatric disorders such as Parkinson's disease, schizophrenia, and drug addiction. Among dopamine receptor subtypes, the D_2 receptor has been studied extensively because most antipsychotics presently in use have high affinity for this receptor (Dixon et al., 1999). It has been postulated that modulation, rather than direct blockade, of the

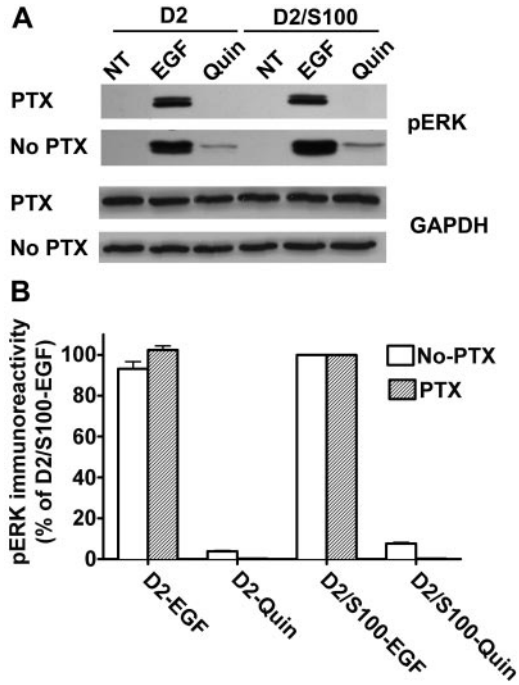

Fig. 6. EGF-induced signaling was independent of D_2 receptor activation of $G\alpha_{i/o}$. Pretreatment with PTX completely prevented activation of ERK by quinpirole (Quin) but had no effect on EGF-induced activation of ERK. HEK293 cells expressing the D_2 receptor alone (D2) or coexpressed with S100B (D2/S100B) were treated with vehicle (NT), EGF (10 ng/ml), or quinpirole (Quin, 1 μM) for 5 min. A, an immunoblot from a representative experiment shows EGF- and quinpirole-induced activation of ERK (pERK), with GAPDH as a loading control, in cells treated with PTX or vehicle (No PTX). B, the results shown are the mean ± S.E. from three independent experiments, expressed as a percentage of the optical density of bands from D2/S100B cells treated with EGF.

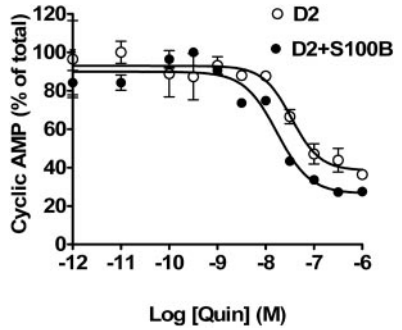

Fig. 7. Coexpression of S100B enhances D_2 receptor inhibition of cAMP accumulation in HEK293 cells. HEK293 cells stably expressing myc-D_2 only or both myc-D_2 and FLAG-S100B were incubated with 30 μM forskolin and increasing concentrations of quinpirole, and cAMP accumulation was determined as described under *Materials and Methods*. The curve shown is representative of three independent experiments.

D_2 receptor might offer the therapeutic benefit without the adverse effects of most antipsychotic drugs (Dixon et al., 1999). One way to achieve this would be to inhibit the binding of dopamine receptor-interacting proteins that enhance D_2 receptor signaling.

Because the third cytoplasmic loop is the primary contact site between G protein-coupled receptors and G proteins, interactions that have been identified between the third cytoplasmic loop of D_2-like receptors and a number of other proteins are likely to influence D_2-like receptor signaling. For example, D_2 and D_3 receptors but not D_1 or D_4 receptors bind the actin-binding protein filamin A, or ABP-280, at a segment in the carboxyl terminus of the third cytoplasmic loop, where both D_2 and D_3 receptors have a potential site of phosphorylation by protein kinase C. D_2 and D_3 receptors expressed in cells that lack ABP-280 have diminished ability to inhibit adenylate cyclase (Li et al., 2000, 2002). Calmodulin (CaM) modulates D_2 receptor signaling by binding to the amino terminal end of the D_2 receptor third cytoplasmic loop (Bofill-Cardona et al., 2000; Liu et al., 2007). Understanding fully how the D_2 receptor functions will clearly require determining the full complement of binding partners for the receptor.

The purpose of this study was to identify and characterize novel binding partners of the dopamine D_2 receptor that might modulate receptor signaling. The B2H system is an efficient *Escherichia coli*-based method for detecting protein-protein interactions in vivo. In this system, detection of protein-protein interactions is based on transcriptional activation of the *HIS3* reporter gene, which allows growth in the presence of 3-AT, a competitive inhibitor of His3 enzyme. Positives are verified using the *aadA* gene, which confers streptomycin resistance, as a secondary reporter. The B2H system offers the ability to screen for binding partners with little background, and using *E. coli* for two-hybrid screening instead of a eukaryotic cell reduces the chance that the host harbors a homolog of one of the interacting protein partners (Joung et al., 2000).

Using the B2H system to screen a rat brain cDNA library, we identified a novel interaction between S100B and the D_2 receptor. The S100 protein family is a highly conserved group of Ca^{2+} binding proteins with molecular masses from 9 to 13 kDa. S100B, a particularly well-characterized member of the S100 family, was first discovered as a major constituent of glia (Moore, 1965); however, it is now known to be expressed in tissues and cell lines including C6 glioma cells, cardiomyocytes, renal tumors, and melanomas (Donato, 1991; Suzushima et al., 1994; Takashi et al., 1994; Zimmer et al., 1997). S100B is a homodimer of 21 kDa, and each S100B subunit contains two EF-hand calcium-binding domains (Zimmer et al., 1997). Although the precise mechanisms for intra- and extracellular functions of S100B are not well understood, processes such as neurite extension, Ca^{2+} flux, cell growth, apoptosis, energy metabolism, and protein phosphorylation are all believed to be modulated in some manner by S100B (Kligman and Hilt, 1988; Donato, 1991; Schäfer and Heizmann, 1996). Most significantly for the proposed interaction with the D_2 receptor, S100B has also been identified in neurons (Ellis et al., 2007). The general model for S100-target protein interactions is similar to that of other Ca^{2+}-binding proteins such as CaM and troponin C; S100B undergoes a conformational change upon binding Ca^{2+} that promotes its interaction with a variety of target proteins (Kligman and Hilt, 1988; Chaudhuri et al., 1997; Drohat et al., 1997).

For further evaluation of the interaction between the D_2 receptor and S100B, we used confocal microscopy to assess the colocalization of endogenous S100B with the neuronal marker MAP2 or with endogenous D_2 receptor in neostriatal neurons. We observed extensive coexpression of S100B and MAP2 as well as S100B and D_2 receptor in our neuronal cultures. We verified the interaction by coimmunoprecipitation of the D_2 receptor with FLAG-S100B from HEK293 cell homogenates and with endogenous S100B from rat neostriatal homogenates. We demonstrated that the third intracellular loop of the D_{2L} and D_{2S} receptors but not D_3 is a contact point for the interaction with S100B using an in vitro histidine-tagged pull-down assay. We also identified an S100B binding motif located at residues 233 to 240 of the D_2 receptor, toward the amino terminus of D_2-IC3 and immediately upstream of the alternatively spliced region, a motif that is not found in the D_3 receptor.

The first signaling pathway identified for D_2-like receptors was inhibition of cAMP accumulation. Another important effector in the D_2 receptor signaling pathway that is potentially regulated by the interaction between the D_2 receptor and S100B is ERK. ERKs belong to the family of mitogen-activated protein kinases, components of parallel protein kinase cascades that transmit signals from a variety of extracellular stimuli to the cell nucleus, thus participating in cell proliferation, differentiation, and survival (Gutkind, 1998). Although the pathway from D_2-like receptors to ERK has not been thoroughly elucidated and may differ depending on cell type and receptor subtype, D_2-like receptor activation of ERK is frequently mediated by pertussis toxin-sensitive G proteins (Welsh et al., 1998; Choi et al., 1999; Wang et al., 2005). Both of these signaling responses to D_2 receptor stimulation were enhanced in cells coexpressing S100B. The mechanism by which S100B enhanced D_2 receptor signaling is unknown. Receptor-interacting proteins frequently alter the trafficking of receptors to the membrane, but preliminary results using cell-surface fluorescence and inhibition of radioligand binding by the membrane-impermeant ligand sulpiride suggest that the abundance of cell-surface receptors was similar in myc-D_{2L}-HEK293 cells and myc-D_{2L}/S100B-HEK293 cells (data not shown). Because S100B is a homodimer, another possibility is that it binds to both the D_2 receptor and to another protein involved in signaling, bringing the two proteins together, but it is not known what other protein might be involved.

Another Ca^{2+}-binding protein, CaM, is important for the activation of ERK by several G protein-coupled receptors (Melien et al., 2002). CaM mediates the activation of ERK by the μ-opioid receptor through a pathway involving the transactivation of the EGF receptor (Belcheva et al., 2001) and by the serotonin 5-HT1A receptor through a process involving agonist-induced receptor internalization (Della Rocca et al., 1999). Our previous work suggests that binding of CaM to the D_2 receptor enhances receptor signaling (Liu et al., 2007). The data presented here suggest that binding of S100B, too, enhances D_2 receptor signaling to both ERKs and cAMP. To our knowledge, this is the first report regarding the interaction of S100B and the D_2 receptor and its role in dopamine D_2 receptor signaling.

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