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D2 and D3 Dopamine Receptor Cell Surface Localization Mediated by Interaction with Protein 4.1N

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ABSTRACT

We identified protein 4.1N as a D2-like dopamine receptor-interacting protein in a yeast two-hybrid screen. Protein 4.1N is a neuronally enriched member of the 4.1 family of cytoskeletal proteins, which also includes protein 4.1R of erythrocytes and the 4.1G and 4.1B isoforms. The interaction of protein 4.1N was specific for the D2 and D3 dopamine receptors and was independently confirmed in pulldown and coimmunoprecipitation assays. Deletion mapping localized the site of dopamine receptor/protein 4.1N interaction to the N-terminal segment of the third intracellular domain of D2 and D3 receptors and the carboxyl-terminal domain of protein 4.1N. D2 and D3 receptors were also found to interact with the highly conserved carboxyl-terminal domain of proteins 4.1R, 4.1G, and 4.1B. Immunofluorescence studies show that protein 4.1N and D2 and D3 dopamine receptors are expressed at the plasma membrane of transfected human embryonic kidney 293 and mouse neuroblastoma Neuro2A cells. However, expression of D2 or D3 receptors with a protein 4.1N truncation fragment reduces the level of D2 and D3 receptor expression at the plasma membrane. These results suggest that protein 4.1N/dopamine receptor interaction is required for localization or stability of dopamine receptors at the neuronal plasma membrane.

Dopamine is the major catecholamine neurotransmitter in mammalian brain and mediates diverse neurological functions, such as regulation of locomotion (Smith et al., 1999), memory formation (Berke and Hyman, 2000), and higher cognitive abilities (Goldman-Rakic, 1996). Dopaminergic signaling is mediated through a small family of G-protein–coupled receptors. Dopamine receptors are divided into two subfamilies, D1-like (D1 and D5) and D2-like (D2, D3, and D4), based on differing amino acid sequence, pharmacologic profiles, and signal transduction pathways (Missale et al., 1998). D1-like receptors couple to stimulatory subsets of heterotrimeric G-proteins and produce increases in cellular cAMP levels, whereas D2-like receptors signal through inhibitory subsets of G-proteins, resulting in inhibition of adenylyl cyclase and lower intracellular cAMP levels (Missale et al., 1998). Aberrant dopaminergic signaling has been implicated in several neuropsychiatric and motor function disorders, such as schizophrenia and Parkinson's disease (Civelli et al., 1993). Although D2-like receptors serve as the major targets of both typical and atypical antipsychotic drugs, the mechanisms underlying alterations in dopaminergic neurotransmission in such neuropathologies as schizophrenia are poorly characterized.

Developing an understanding of how neurotransmitter receptor signaling is regulated has become a central focus in molecular neurobiology. Results from many different laboratories have pointed to protein-protein interactions as a key determinant in the regulation of neurotransmitter receptor function. Many of these interactions have been elucidated using the yeast two-hybrid system, in which segments of a receptor are used to fish out interacting proteins from brain cDNA libraries. An example of the complexity of protein-protein interactions has recently been described for the *N*-methyl-D-aspartate receptor (Husi et al., 2000). Using a proteomic-based approach, the *N*-methyl-D-aspartate–subtype of glutamate receptors has been shown to be part of a multiprotein complex consisting of more than 70 unique proteins that include cytoskeletal proteins, scaffolding and adaptor proteins, cell-adhesion molecules, and signaling proteins (Husi et al., 2000).

Using conventional two-hybrid screens, several dopamine receptor interacting proteins have now been identified. These include the D1-interacting proteins, calcyon (Lezcano et al.,

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; 4.1N, protein 4.1N; 4.1R, erythrocyte band 4.1; CTD, carboxyl-terminal domain; D2S, dopamine receptor D2 short subtype; D2L, dopamine receptor D2 long subtype; IC3, third intracellular domain of dopamine receptors; FLN-A, filamin-A; cPLA$_2$, cytosolic phospholipase A$_2$; GST, glutathione *S*-transferase; MBD, membrane-binding domain; SABD, spectrin actin-binding domain; GluR, glutamate receptor; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein.

2000) and DRiP78 (Bermak et al., 2001), and the D2-interacting proteins, spinophilin (Smith et al., 1999) and filamin-A (Li et al., 2000; Lin et al., 2001). We have now identified protein 4.1N as an additional D2-like–interacting protein. Protein 4.1N is a recently identified member of the 4.1 family of cytoskeletal-associated proteins and is specifically enriched in neurons (Walensky et al., 1999). The 4.1 proteins are critical components of the spectrin-actin cytoskeleton and provide attachment between the cytoskeleton and the cell membrane. Protein 4.1N has been shown to directly interact with the GluR1 subunit of the AMPA receptor and to colocalize with AMPA receptors at excitatory synapses (Shen et al., 2000). Among dopamine receptors, protein 4.1N interacts specifically with D2 and D3 dopamine receptors. Coxpression of D2 or D3 dopamine receptors with a mutant protein 4.1N that contains the dopamine receptor binding site but lacks the 4.1N membrane-binding domain decreases cell surface expression of D2 and D3 in transfected mouse neuroblastoma Neuro2A and human embryonic kidney (HEK) 293 cells. Our data suggest an important functional role for dopamine receptor/4.1N interaction in the localization or stability of dopamine receptors at the neuronal cell surface.

Materials and Methods

DNA Constructs and Protein Interaction Assays. All constructs were generated by polymerase chain reaction amplification and verified by DNA sequence analysis. A segment of the third intracellular (IC3) domain of D2L (amino acids 211–306) was constructed in the GAL4 DNA-binding domain expression vector, pAS2-1 (BD Biosciences Clontech, Palo Alto, CA) and used to screen a human brain cDNA library subcloned in the GAL4-activation domain vector pACT2 (BD Biosciences Clontech). Bait and prey plasmids were simultaneously cotransformed into the yeast strain MaV103 as described previously (Lin et al., 2001). A total of 1.7×10^6 independent clones were screened by growth on Leu^-/Trp^-/His^-/Ura^- selection plates. Protein interaction was assayed by the β-galactosidase activity assay as described previously (Lin et al., 2001). Additional constructs encoding the IC3 domains of D1, D3, D4, and D5 dopamine receptors, as well as M1 muscarinic and β2-adrenergic receptors, were also constructed in pAS2-1.

To map sites within D2, D3, and protein 4.1N that contribute to dopamine receptor/4.1N interaction, truncated segments of the D2 and D3 IC3 domains were constructed in pAS2-1, and segments of protein 4.1N were inserted into pACT2. Bait and prey plasmids were simultaneously transformed into the yeast strain MaV103, and interactions were determined using the β-galactosidase activity assay.

Cell Culture and Transfection. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. HEK 293 cells stably expressing FLAG-tagged D2 receptors (HEK 293/D2) were generously provided by Dr. Mark von Zastrow (University of California San Francisco). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 300 μg/ml Geneticin (Invitrogen). Cells were transiently transfected with LipofectAMINE 2000 transfection reagent (Invitrogen) under conditions recommended by the manufacturer. Neuro2A cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 1.0 mM sodium pyruvate, and 0.1 mM nonessential amino acids (Cellgro, Herndon, VA). Neuro2A cells were transiently transfected with Tfx-50 transfection reagent (Promega, Madison, WI) according to the manufacturer's instructions.

Glutathione *S*-Transferase Pulldown and Coimmunoprecipitation. The IC3 domains of D2S (residues 211–344), D2L (residues 211–373), and D3 (residues 211–329) were constructed in the expression vector pGEX-4T-1 (Amersham Biosciences Inc., Piscataway, NJ) to generate fusion proteins D2S-GST, D2L-GST, and D3-GST, respectively. Fusion proteins were induced in *Escherichia coli* strain BL21 and purified using glutathione-Sepharose as described by the manufacturer (Amersham Biosciences Inc.). Full-length protein 4.1N was constructed in the pEGFP-C2 expression vector (BD Biosciences Clontech) to generate plasmid 4.1N-EGFP. EGFP-tagged protein 4.1N was transiently expressed in HEK 293T cells, and total cell lysates were prepared 24 h after transfection. Binding assays were carried out as described previously (Lin et al., 2001). Eluted proteins were separated by SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with either a 1:200 dilution of a rabbit polyclonal anti-GFP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a 1:2500 dilution of an anti-4.1N monoclonal antibody (BD Biosciences PharMingen, San Diego, CA) and developed with horseradish peroxidase-conjugated goat anti-rabbit (1:2000) or goat anti-mouse (1:10,000) secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Immunoreactivity was detected by enhanced chemiluminescence with an ECL Plus kit (Amersham Biosciences Inc.).

For coimmunoprecipitation, total cell lysates were prepared from HEK 293T cells transiently transfected with FLAG-tagged D3 (D3-FLAG) receptors and EGFP-tagged protein 4.1N. D3 receptors were immunoprecipitated with the anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO) as described previously (Karpa et al., 2000). Immunocomplexes were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed using a rabbit anti-GFP antibody (1:200; Santa Cruz Biotechnology, Inc.). Proteins were visualized using peroxidase-conjugated goat anti-rabbit secondary antibodies (1:2000; Jackson Immunoresearch Laboratories, Inc.).

Immunofluorescence. HEK 293T cells were transiently cotransfected with plasmids encoding either EGFP-tagged protein 4.1N and FLAG-tagged D3 receptors or myc-tagged protein 4.1N and FLAG-tagged D2L receptors. Cells were fixed in acetone/methanol (1:1, v/v) 24 h after transfection, permeabilized with 0.03% Triton X-100, and stained with either a goat polyclonal anti-myc antibody (1:1000; Santa Cruz Biotechnology, Inc.) or a 1:1000 dilution of anti-FLAG M2 monoclonal antibody. Endogenously expressed protein 4.1N was detected with a 1:200 dilution of a monoclonal anti-4.1N antibody (BD Biosciences PharMingen). Staining was visualized with either rhodamine red-conjugated goat anti-mouse or rabbit anti-goat secondary antibodies (Jackson Immunoresearch Laboratories, Inc.). GFP fluorescence was detected directly by fluorescence microscopy. Immunofluorescence was visualized by confocal laser microscopy using a Zeiss LSM 210 confocal microscope (Carl Zeiss GmbH, Jena, Germany).

Cell Surface Expression of Dopamine Receptors. HEK 293/D2 cells were transiently transfected with an EGFP-tagged protein 4.1N truncation fragment (amino acids 712–880). Cell surface proteins were biotinylated 24 h after transfection with 1 mg/ml sulfo-NHS-SS-Biotin (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. Crude membranes were prepared, and immunoprecipitations were performed as described previously (Karpa et al., 2000). Immunoblots containing biotinylated proteins were probed using the VectaStain ABC detection system (Vector Laboratories, Burlingame, CA), and staining was detected by enhanced chemiluminescence. Immunoblots were quantitated using a laser densitometer (Molecular Dynamics, Sunnyvale, CA) and analyzed using the Quantity One software package (PDI, Inc., Huntington Station, NY). Membrane protein levels were normalized to the cell surface marker, the Na^+, K^+-ATPase α1 subunit, which was detected with an anti-NaK-ATPase α1 subunit monoclonal antibody (Upstate Biotechnology, Lake Placid, NY).

Results

Interaction of Protein 4.1N with D2 and D3 Dopamine Receptors. To identify proteins that interact with the D2-like family of dopamine receptors, we used the IC3 domain of the D2L dopamine receptor as bait to screen an adult human brain cDNA library. Of the 1.7×10^6 clones screened, we isolated two clones containing the complete open reading frame of protein 4.1N, a neuronal homolog of the erythrocyte membrane cytoskeletal protein 4.1 (4.1R). Protein 4.1N has been implicated in the stability and plasticity of the neuronal membrane via protein-protein interactions (Walensky et al., 1999). To examine the specificity of the protein 4.1N interaction with D2 receptors, we used the yeast two-hybrid system to test the interaction of protein 4.1N with additional dopamine and G-protein-coupled receptors. Bait constructs encoding the IC3 domains of D1, D2S, D3, D4, and D5 dopamine receptors, as well as the M1-muscarinic receptor and β2-adrenergic receptor, were tested for an interaction with protein 4.1N. We found that interaction with protein 4.1N was restricted to D2 (D2S and D2L) and D3 receptors. Based on the intensity of the β-galactosidase colorimetric assays, protein 4.1N showed a stronger interaction with the D3 dopamine receptor than the D2S or D2L dopamine receptors (data not shown). Protein 4.1N did not interact with the IC3 domain of D1, D4, or D5 dopamine receptors or the M1 muscarinic or β2-adrenergic receptor. These results suggest that, among dopamine receptors, protein 4.1N specifically interacts with the D2 and D3 subtypes.

To verify the interaction of protein 4.1N with D2 and D3 dopamine receptors, we tested the ability of protein 4.1N to associate with GST fusion proteins containing the D2S, D2L, or D3 IC3 domains (D2S-GST, D2L-GST, and D3-GST, respectively). As shown in Fig. 1A, a Western blot containing lysate prepared from HEK 293T cells expressing EGFP-tagged protein 4.1N (4.1N-EGFP) produced a band of ~160 kDa that was immunoreactive with anti-GFP antibodies. This band represents 4.1N-EGFP expressed in transfected HEK 293T cells. The same band was detected in pulldown assays after the cell lysate was incubated with D2S-GST, D2L-GST, or D3-GST fusion proteins but not with GST alone.

The interaction of protein 4.1N with the D3 dopamine receptor was also tested by coimmunoprecipitation experiments. To demonstrate interaction, we tested the ability of an anti-FLAG monoclonal antibody to coimmunoprecipitate D3 dopamine receptors and protein 4.1N from HEK 293T cells transiently expressing D3-FLAG and 4.1N-EGFP. As shown in Fig. 1B, anti-GFP antibodies were reactive with a band of ~160 kDa in cell lysates and immunocomplexes. This band represents 4.1N-EGFP. Protein 4.1N was not immunoprecipitated from cells that were transfected with either D3-FLAG or 4.1N-EGFP alone. These studies provide strong evidence for an association between protein 4.1N and D3 dopamine receptors in mammalian cells. Although we were able to demonstrate D2 receptor/protein 4.1N interaction in pulldown assays, we were unable to coimmunoprecipitate D2 receptors and protein 4.1N from transfected cells. Our inability to coimmunoprecipitate these two proteins may reflect a weaker interaction between D2 receptors and protein 4.1N than between protein 4.1N and D3 receptors.

Mapping Protein-Protein Interaction Domains. We carried out deletion mapping studies to determine the domains within D2 and D3 dopamine receptors that contribute to dopamine receptor/protein 4.1N interaction. Truncated fragments of the D2S, D2L (Fig. 2A), and D3 (Fig. 2B) IC3 domains were tested for interaction with full-length protein 4.1N using the yeast two-hybrid system. Constructs 2 (residues 211–306), 5 (residues 211–270), and 6 (residues 211–241) tested positive in the β-galactosidase assay. Constructs 3 (residues 292–373) and 4 (residues 230–313) did not interact with protein 4.1N. These results indicate that amino acids 211 to 241 of the D2 IC3 domain encompass the protein 4.1N binding region. This region is identical in the D2S and D2L receptors. Truncation studies were also performed with the IC3 domain of D3. Constructs 8 (residues 211–240) and constructs 11 through 14 (residues 211–240, 211–236, 211–233, and 211–230, respectively) were capable of positive interaction with protein 4.1N. However, constructs 9 (residues 240–329) and 10 (residues 227–240) failed to demonstrate a positive interaction. From the D3 truncation studies we conclude that the most N-terminal segment of the IC3 domain of D3 (residues 211–230) contains the protein 4.1N binding site. The protein 4.1N binding region is 52% similar between the D2 and D3 receptors, with the highest level of similarity

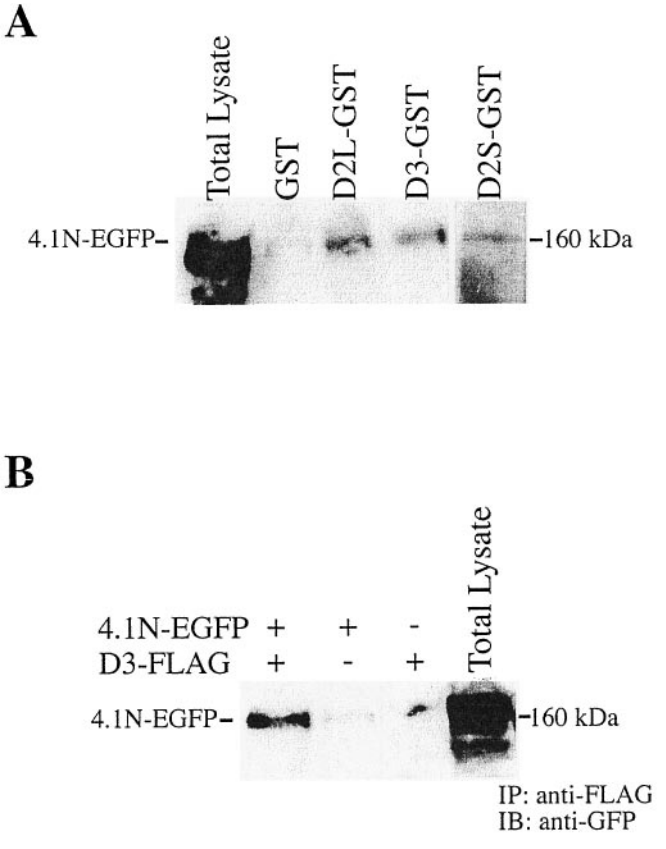

Fig. 1. Protein 4.1N associates with D2 and D3 dopamine receptors. A, fusion proteins were used to pull down EGFP-tagged protein 4.1N from HEK 293T cell lysates. Protein 4.1N was pulled down in the presence of D2S-GST, D2L-GST, or D3-GST fusion proteins but not with GST alone. B, coimmunoprecipitation of D3 receptors with protein 4.1N. Anti-FLAG monoclonal antibody was used to immunoprecipitate D3 receptors from lysates prepared from HEK 293T cell lysates transiently expressing EGFP-tagged protein 4.1N and FLAG-tagged D3 dopamine receptors. A Western blot containing cell lysates was probed with an anti-GFP antibody. Protein 4.1N was detectable in lysates prepared from cells cotransfected with protein 4.1N and D3 receptors but not in lysates from cells expressing protein 4.1N or D3 receptors alone.

(92%) spanning residues 211 to 222 of the D2/D3 receptor IC3 domain (Fig. 2C).

To map the domain within protein 4.1N that interacts with the dopamine receptors, we carried out interaction assays of truncated constructs of protein 4.1N and the IC3 domains of the D2S, D2L, and D3 receptors (Fig. 3). We initially generated constructs to encompass the various conserved regions of protein 4.1N (Fig. 3) and found that only the truncation fragment containing the carboxyl-terminal domain (CTD; residues 440–880) was capable of interaction with D2/D3; fragments containing the membrane-binding domain (MBD) and the spectrin actin-binding domain (SABD) were not sufficient for D2/D3 interaction (residues 1–440 and 207–662, respectively). A fragment encoding only the CTD (residues 712–880) was also capable of interaction, whereas a truncation fragment lacking the CTD (residues 1–712) was incapable of D2/D3 interaction. Residues 712 through 824 of protein 4.1N failed to interact with D2/D3, suggesting that the most C-terminal region of the CTD is necessary for D2/D3 interaction and that both D2 and D3 share the same binding domain within protein 4.1N.

The 4.1 family of proteins is comprised of four isoforms, 4.1N, 4.1R, 4.1G, and 4.1B. The 4.1 proteins share three conserved domains, a MBD, a SABD, and a conserved CTD (Hoover et al., 2000). Among the 4.1 proteins, the CTD exhibits 60 to 85% amino acid sequence similarity with the highest homology within the C-terminal portion (Fig. 4). Because we localized the D2/D3 binding site to this segment of protein 4.1N, we used the yeast two-hybrid assay to test whether the IC3 domains of D2 and D3 receptors interacted with other protein 4.1 family members. Bait constructs encoding the IC3 domains of D2 and D3 dopamine receptors were tested for interaction with the CTD of 4.1R (residues 723–858), 4.1G (residues 853–989), and 4.1B (residues 812–951). D2/D3 constructs were found to interact with the CTD of all 4.1 family members (data not shown). Interaction with protein 4.1 family members may thus provide a general mechanism for linking D2 and D3 receptors to the neuronal cytoskeleton.

Protein 4.1N and D2/D3 Dopamine Receptors Are Coexpressed at the Plasma Membrane. To further characterize protein 4.1N/dopamine receptor interaction, we examined expression of protein 4.1N and D2/D3 receptors in transfected HEK 293T cells. As shown in Fig. 5, FLAG-tagged D2L receptors (Fig. 5A) and myc-tagged protein 4.1N (Fig. 5B) were coexpressed at the plasma membrane of HEK 293T cells (Fig. 5C). FLAG-tagged D3 receptors (Fig. 5D) and EGFP-tagged protein 4.1N (Fig. 5E) also showed plasma membrane coexpression (Fig. 5F). Similar results were obtained in transfected Neuro2A cells (data not shown).

We also examined the localization of D2 receptors and protein 4.1N endogenously expressed in HEK 293/D2 cells. FLAG-tagged D2 receptors (Fig. 5G) and endogenously expressed protein 4.1N (Fig. 5H) exhibited coexpression (Fig. 5I) in these cells. This was compared with the expression of D2 receptors and the cytosolic phospholipase A_2 ($cPLA_2$)-interacting protein, a protein previously identified in a yeast two-hybrid screen as interacting with D2 receptors (Lin, 2001). In transfected HEK 293T cells, FLAG-tagged D2 receptors were detected at the plasma membrane as well as in the cytoplasm (Fig. 5J), whereas EGFP-tagged $cPLA_2$-interacting protein was detected exclusively in the nucleus (Fig. 5K). The merged image (Fig. 5L) clearly shows that the two proteins do not colocalize within the cell. Together, our results indicate that D2/D3 receptors and protein 4.1N are coexpressed at the plasma membrane of cultured cells. Co-

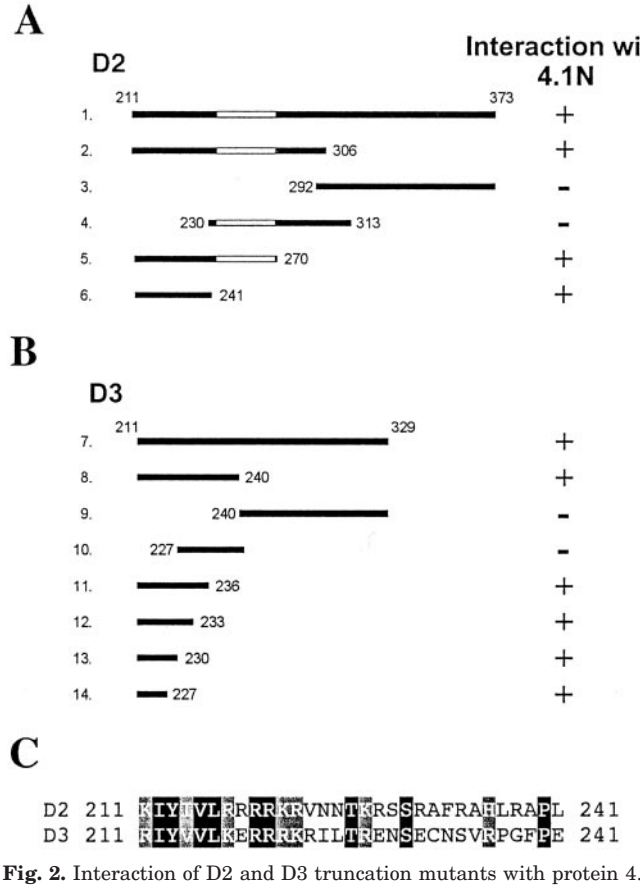

Fig. 2. Interaction of D2 and D3 truncation mutants with protein 4.1N. Schematic representation of D2 (A) and D3 (B) IC3 domain constructs tested for interaction with protein 4.1N in the two-hybrid assay. Interaction with protein 4.1N is indicated by the presence (+) or absence (−) of β-galactosidase activity. The alternatively spliced 29-amino acid exon in D2L is indicated by the open box. C, amino acid sequence alignment of the protein 4.1N binding site within the IC3 domain of the D2 and D3 receptors. Identical amino acids are depicted by black boxes, and conserved amino acids are depicted by gray boxes.

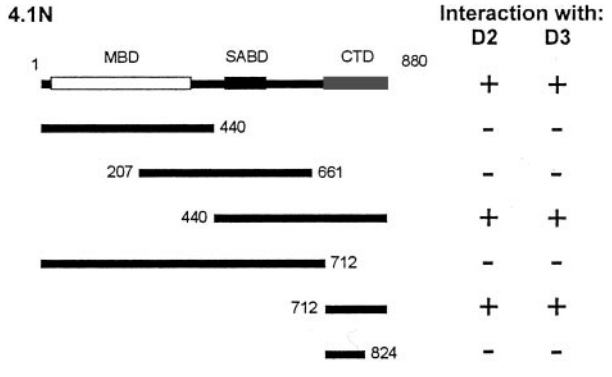

Fig. 3. Mapping the D2 and D3 receptor binding site on protein 4.1N. A schematic representation of constructs encoding truncations of protein 4.1N. Constructs were tested for interaction with fragments of the D2 (residues 211–241) or D3 (residues 211–227) IC3 domain containing the protein 4.1N binding site. Interaction with the D2 or D3 receptor is indicated by the presence (+) or absence (−) of β-galactosidase activity. The MBD is represented by the open box, the SABD is represented by the black box, and the CTD is depicted by the shaded box.

expression of D2/D3 receptors and protein 4.1N within the same cellular compartment is consistent with the idea that dopamine receptors and protein 4.1N are capable of interaction in mammalian cells.

Protein 4.1N Is Required for D2 and D3 Dopamine Receptor Cell Surface Expression. To help determine the physiological significance of protein 4.1N/dopamine receptor interaction, we transfected a truncated protein 4.1N construct into murine Neuro2A cells. Neuro2A cells endogenously express protein 4.1N, which is localized predominantly at the cell surface (data not shown). The protein 4.1N truncation fragment (amino acid residues 712–880) contains the dopamine receptor binding site but lacks the MBD and the SABD. When overexpressed in Neuro2A cells, the protein 4.1N truncation fragment should act in a dominant-negative fashion and compete with endogenously expressed protein 4.1N for dopamine receptor binding. Because the protein 4.1N truncation fragment cannot bind to the plasma membrane or the submembranous cytoskeleton, D2 or D3 receptors that bind to the truncation fragment should also fail to localize to the cell surface. As shown in Fig. 6, transiently transfected FLAG-tagged D2 (Fig. 6A) or D3 (Fig. 6D) receptors were detected predominantly at the plasma membrane of Neuro2A cells. The cell-surface distribution of the D2 (Fig. 6B) and D3 (Fig. 6E) receptors was not altered when coexpressed with EGFP-tagged protein 4.1N, indicating that overexpression of protein 4.1N did not affect the normal plasma membrane distribution of D2 or D3 receptors. However, when FLAG-tagged D2 or D3 receptors were cotransfected with the protein 4.1N truncation fragment, D2 (Fig. 6C) and D3 (Fig. 6F) receptor staining at the cell surface was significantly reduced. Similar results were obtained using HEK 293/D2 cells (data not shown). These results suggest that protein 4.1N/dopamine receptor interaction is required for the proper targeting or stabilization of D2 and D3 receptors at the plasma membrane.

We also used a cell surface biotinylation assay to analyze the effect of the protein 4.1N truncation fragment on the levels of D2 receptors expressed at the plasma membrane. To do this, HEK 293/D2 cells were transiently transfected with the protein 4.1N truncation fragment, and cell surface proteins were biotinylated. Biotinylated membrane proteins were immunoprecipitated using an anti-FLAG monoclonal antibody, and immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and quantitated

Fig. 4. Comparison of the CTD sequences among protein 4.1 family members. Alignment of the CTDs of protein 4.1R (residues 723–858), 4.1G (residues 853–989), 4.1B (residues 812–951), and 4.1N (residues 712–880). Amino acid numbers are shown at the left. Identical amino acids are within the black boxes, and conserved amino acids are within the gray boxes.

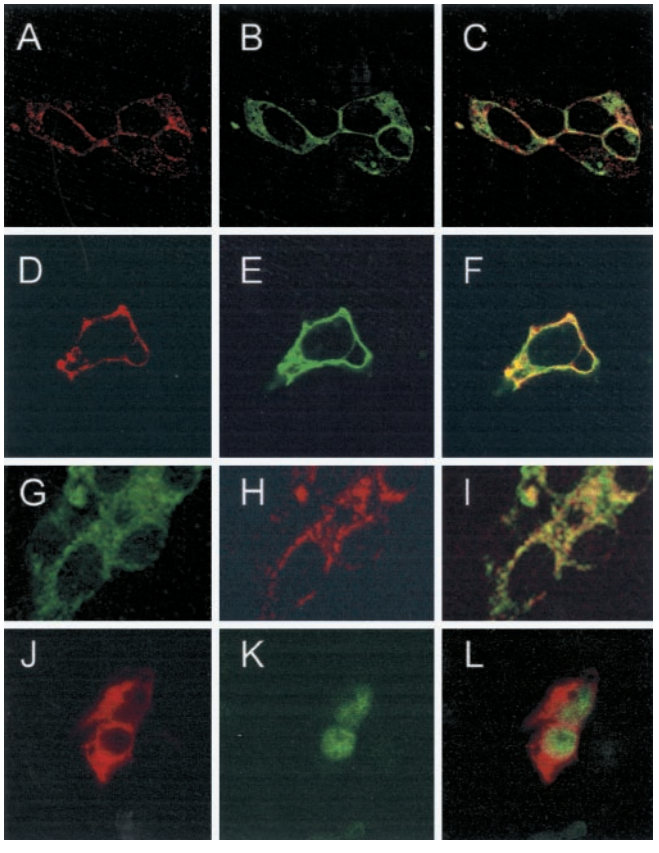

Fig. 5. Protein 4.1N and D2/D3 dopamine receptors are coexpressed at the plasma membrane. HEK 293T cells were transiently transfected with FLAG-tagged D2L and myc-tagged protein 4.1N. Confocal images of FLAG-D2L expression (A), myc-protein 4.1N expression (B), and a merged image of D2L and 4.1N expression (C). HEK 293T cells transiently expressing FLAG-tagged D3 receptors (D), EGFP-tagged protein 4.1N (E), and a merged image of D3 and protein 4.1N expression (F). HEK 293/D2 cells stained for FLAG-tagged D2 receptors (G) and endogenous protein 4.1N (H). Merged image of D2 receptors and endogenous protein 4.1N (I). HEK 293T cells transiently expressing FLAG-tagged D2 receptors (J), EGFP-tagged $cPLA_2$-interacting protein (K), and a merged image of D2 receptors and $cPLA_2$-interacting protein (L).

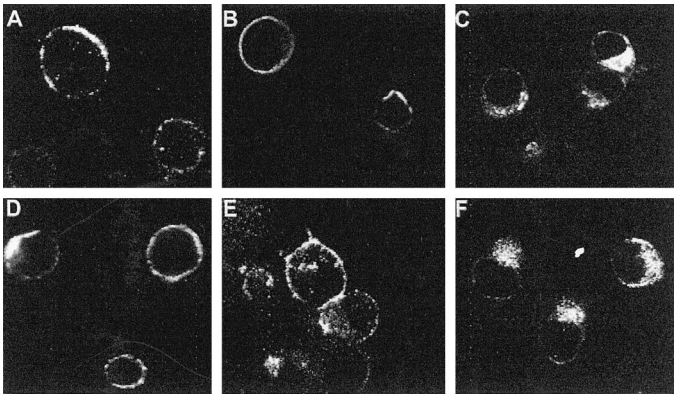

Fig. 6. Interaction between protein 4.1N and D2/D3 receptors is required for D2/D3 receptor cell surface expression. Expression of proteins was detected by confocal laser microscopy. Cell surface expression of FLAG-tagged D2 (A) and D3 (D) receptors in Neuro2A cells. Neuro2A cells cotransfected with EGFP-tagged protein 4.1N and either FLAG-tagged D2 (B) or D3 (E) receptors stained with anti-FLAG antibodies. Coexpression in Neuro2A cells of FLAG-tagged D2 (C) or D3 (F) receptors with an EGFP-tagged protein 4.1N CTD truncation fragment. In the presence of the 4.1N truncation fragment, D2 and D3 receptors show decreased expression at the plasma membrane.

by laser densitometry. As shown in Fig. 7, expression of the protein 4.1N truncation fragment produced an ~50% decrease in the numbers of cell surface D2 receptors compared with untransfected HEK 293/D2 cells endogenously expressing protein 4.1N. These experiments further support the idea that the protein 4.1N/dopamine receptor interaction is required for the proper targeting or stabilization of D2 receptors at the plasma membrane.

Protein 4.1N and Filamin-A Simultaneously Bind D2 Dopamine Receptors. We have previously established an interaction between the actin cross-linking protein filamin-A (FLN-A) and the D2 and D3 dopamine receptors (Lin et al., 2001). Our mapping studies indicate that FLN-A and protein 41N bind to a common region with the IC3 domain (residues 211–241) of the D2 receptor. We therefore sought to determine whether FLN-A and protein 4.1N can simultaneously bind to the D2 receptor or whether they compete for binding. To address this question, we overexpressed EGFP-tagged protein 4.1N in HEK 293/D2 cells. These cells endogenously express protein 4.1N (Fig. 5H) and FLN-A (Lin et al., 2001). Crude membrane proteins were prepared from transfected cells and immunoprecipitated with an anti-D2 antibody. Immunocomplexes were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with a monoclonal anti-FLN-A antibody. As shown in Fig. 8, overexpression of protein 4.1N did not reduce the levels of FLN-A associated with D2 receptors in HEK 293/D2 membrane preparations. These results are most consistent with the idea that FLN-A and protein 4.1N can simultaneously bind to the D2 receptor and together may strengthen or stabilize attachment of dopamine receptors to the cytoskeleton. It will be of interest to determine whether FLN-A and protein 4.1N interact with the same or different residues within the common binding domain of the D2 receptor.

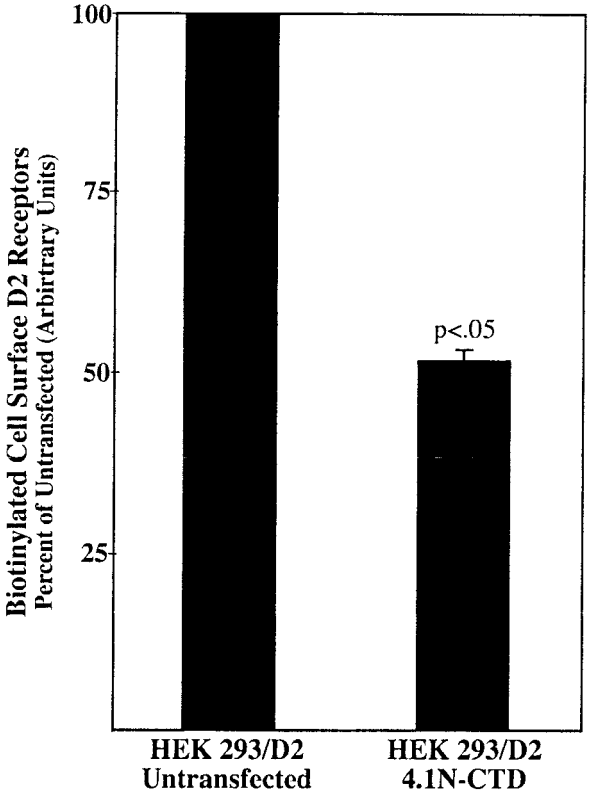

Fig. 7. Expression of the protein 4.1N CTD truncation fragment causes a decrease in cell surface D2 receptors. HEK 293/D2 cells were transiently transfected with the protein 4.1N CTD truncation fragment (amino acids 712–880). Cell surface proteins were biotinylated, and FLAG-tagged D2 receptors were immunoprecipitated with a monoclonal anti-FLAG antibody. Biotinylated receptors were quantitated by laser densitometry. Cells expressing the protein 4.1N truncation fragment show an ~50% decrease in the level of cell surface D2 receptors (Student's two-tailed t test, $n = 3$, $p < 0.05$) compared with untransfected cells expressing endogenous protein 4.1N

Discussion

We have identified protein 4.1N as a protein that interacts with the D2 and D3 dopamine receptor subtypes. Protein 4.1N is a member of the 4.1 family of cytoskeletal proteins. This family includes the prototype erythrocyte protein 4.1R, an abundant protein of the human erythrocyte membrane (Tyler et al., 1979), and the paralogous vertebrate proteins 4.1G and 4.1B (Parra et al., 1998, 2000). In erythrocytes, protein 4.1R plays a critical role in the organization and maintenance of the spectrin-actin cytoskeleton and attachment of the cytoskeleton to the plasma membrane via interaction with integral membrane components, such as glycophorin C and the band 3 anion transporter (Tyler et al., 1979; Pasternack et al., 1985). Structurally, protein 4.1 family members share three conserved domains: a membrane-binding domain, a spectrin actin-binding domain, and a carboxyl-terminal domain of unknown function (Hoover and Bryant, 2000). The presence of these conserved structural features suggests that the 4.1 family members may share a common functional role in linking the spectrin-actin cytoskeleton to a variety of binding partners, including components of signal transduction pathways (Hoover and Bryant, 2000). Indeed, protein 4.1N has recently been shown to interact with the GluR1 subunit of the AMPA receptor, leading to the idea that protein 4.1N/AMPA receptor interaction may provide a mechanism that links AMPA receptors to the spectrin-actin cytoskeleton (Shen et al., 2000).

We have mapped the binding sites on protein 4.1N and the D2 and D3 dopamine receptors that are responsible for protein 4.1N/dopamine receptor interaction. The dopamine receptor binding site on protein 4.1N maps to the CTD. The CTD is highly conserved among all 4.1 family members, and both D2 and D3 receptors were also found to interact with the CTDs of proteins 4.1R, 4.1G, and 4.1B. The GluR1 subunit of the AMPA receptor has also been shown to interact with the CTD of proteins 4.1N and 4.1G (Shen et al., 2000), suggesting that sequences within the CTD of 4.1 proteins may constitute

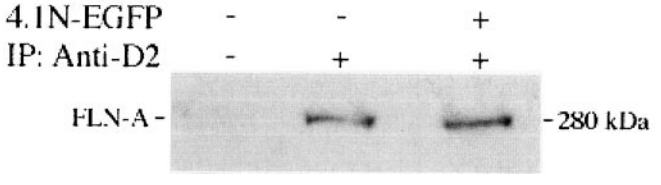

Fig. 8. Protein 4.1N and filamin-A simultaneously bind D2 receptors. HEK 293/D2 cells were transiently transfected with EGFP-tagged protein 4.1N (4.1N-EGFP). Crude membranes were solubilized and immunoprecipitated with an anti-D2 polyclonal antibody (Santa Cruz Biotechnology, Inc.). Immunocomplexes were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with an anti-FLN-A monoclonal antibody (1:1000; Chemicon International, Temecula, CA). The position of the 280-kDa filamin-A monomer is shown.

a novel protein-interaction motif. Truncation analysis localized a region within the N-terminal portion of the D2 (residues 211–241) and D3 (residues 211–227) receptor IC3 domains that constitute the protein 4.1N binding site. This segment has also been identified as an interaction site for the actin cross-linking protein, FLN-A (Lin et al., 2001), and the small calcium binding protein calmodulin (Bofill-Cardona et al., 2000). Together, these results suggest that sequences within the N-terminal portion of the D2 and D3 receptor IC3 domain may represent a previously unrecognized protein interaction domain.

The identification of the D2/D3 binding site on protein 4.1N has allowed us to design a strategy to analyze the potential significance of the protein 4.1N/dopamine receptor interaction. Our approach was to overexpress a fragment of protein 4.1N containing the CTD, which is the dopamine receptor-binding domain, but lacking the membrane- or spectrin actin-binding domains. This construct acts in a dominant-negative fashion by competing with endogenous protein 4.1N for dopamine receptor binding in Neuro2A and HEK 293 cells. By overexpressing the 4.1N fragment, most of the transfected D2 and D3 receptors are predicted to bind to the 4.1N fragment and not to endogenous protein 4.1N. Using this approach, we found that the protein 4.1N truncation fragment had a significant effect on the cellular distribution of D2 and D3 receptors in transfected Neuro2A and HEK 293 cells. In the absence of the protein 4.1N truncation fragment, transfected D2 or D3 dopamine receptors were localized predominantly at the plasma membrane of transfected cells. In the presence of the 4.1N fragment, however, the level of dopamine receptor expression at the plasma membrane was decreased by ~50%. In an analogous approach, Shen et al. (2000) demonstrated that overexpression of the CTDs of proteins 4.1N and 4.1G attenuated cell surface expression of the GluR1 AMPA receptor subunit. Our results provide support for the idea that protein 4.1N serves to link D2 and D3 dopamine receptors to the cytoskeleton and is required for the plasma membrane localization or stability of these neurotransmitter receptors.

The prototypical member of the protein 4.1 family, 4.1R, was originally identified as a component of the erythrocyte membrane (Tyler et al., 1979). Analysis of the expression profile of protein 4.1 family members indicates that each has a unique expression pattern in mammalian brain (Walensky et al., 1998, 1999; Shi et al., 1999; Hoover and Bryant, 2000; Parra et al., 2000; Scott et al., 2001). In cortical neurons, D2 dopamine receptors and protein 4.1N colocalize in many neurons; however, there are nonneuronal cells that express D2 receptors but not protein 4.1N. It is possible that other 4.1 family members are expressed in these cells and provide sites of attachment for D2 receptors. In support of this idea, our data indicate that D2 and D3 receptors interact with proteins 4.1R, 4.1G, and 4.1B in the two-hybrid system. It will be important to determine whether these interactions can be validated using the combination of biochemical and cell biological approaches used to confirm protein 4.1N/dopamine receptor interaction. If D2 and D3 receptors do in fact interact with each of the protein 4.1 family members, it will be of interest to learn whether all 4.1 proteins perform a similar function in terms of anchoring dopamine receptors to the cytoskeleton. The differential distribution of 4.1 family members in brain suggests that each protein 4.1 subtype may serve to anchor dopamine receptors to the cytoskeleton in a cell- or region-specific fashion. It is possible that different 4.1 proteins could also play an important role in the targeting to or stability of dopamine receptors at specific subcellular membrane domains. Disruption of the interaction between dopamine receptors and each of the protein 4.1 family members will be needed to address these important issues.

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