Nucleocytoplasmic Shuttling of Dysbindin-1, a Schizophrenia-related Protein, Regulates Synapsin I Expression

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Dysbindin-1 is a 50-kDa coiled-coil-containing protein encoded by the gene DTNBP1 (dystrobrevin-binding protein 1), a candidate genetic factor for schizophrenia. Genetic variations in this gene confer a susceptibility to schizophrenia through a decreased expression of dysbindin-1. It was reported that dysbindin-1 regulates the expression of presynaptic proteins and the release of neurotransmitters. However, the precise functions of dysbindin-1 are largely unknown. Here, we show that dysbindin-1 is a novel nucleocytoplasmic shuttling protein and translocated to the nucleus upon treatment with leptomycin B, an inhibitor of exportin-1/CRM1-mediated nuclear export. Dysbindin-1 harbors a functional nuclear export signal necessary for its nuclear export, and the nucleocytoplasmic shuttling of dysbindin-1 affects its regulation of synapsin I expression. In brains of sandy mice, a dysbindin-1-null strain that displays abnormal behaviors related to schizophrenia, the protein and mRNA levels of synapsin I are decreased. These findings demonstrate that the nucleocytoplasmic shuttling of dysbindin-1 regulates synapsin I expression and thus may be involved in the pathogenesis of schizophrenia.

Dysbindin-1, encoded by the gene DTNBP1 (dystrobrevin-binding protein 1), was originally identified in a yeast two-hybrid screen as a partner that binds to α- and β-dystrobrevin (1). The polymorphisms in DTNBP1 are associated with schizophrenia (2–16). These variations confer a susceptibility to schizophrenia through a decreased expression of dysbindin-1 (17–19). The expression of dysbindin-1 is decreased in schizophrenic brains, especially in the hippocampal formation (HF) (20–23). sandy mice, which do not express dysbindin-1 protein due to a deletion in DTNBP1 (24), show impaired long term memory retention and working memory (25, 26) and also display abnormal behaviors related to schizophrenia (27–31), implying that dysbindin-1 plays important roles in schizophrenia.

Dysbindin-1 is widely and abundantly expressed in rodent and human brains and has important functions in the cytoplasm (1, 20, 32). It is a member of the BLOC-1 (biogenesis of lysosome-related organelles complex 1) and controls synaptic homeostasis (24, 33–36). In the HF, dysbindin-1 is located in postsynaptic densities and synaptic vesicles of neurons (37). In postsynaptic neurons, dysbindin-1 is thought to be involved in one or more postsynaptic density functions in trafficking and tethering of receptors and signal transduction proteins (31, 38–40). In presynaptic neurons, a decrease of dysbindin-1 is associated with glutamatergic alterations (20). In cultured cells, it regulates the expression of some presynaptic proteins as well as the release of glutamate or dopamine (6, 41). Several studies have shown that a decreased expression of presynaptic proteins may be a characteristic of schizophrenia (42–44). Among these presynaptic proteins, synapsins are a family of synaptic vesicle-associated phosphoproteins that are involved in the regulation of neurotransmitter release (45). The protein level of total synapsins is decreased significantly in the HF in patients with schizophrenia (46). In the synapsin family, synapsin I is lower in the HF of some schizophrenic brains (42). Synapsin II (47) and synapsin III (48) are also decreased in the prefrontal cortex in subjects with schizophrenia. Interestingly, it was reported that dysbindin-1 regulates the expression of synapsin I in rat primary cortical neuronal cultures (6). However, the mechanism by which dysbindin regulates synapsin expression remains largely unknown.

Here we identify that dysbindin-1 is a nucleocytoplasmic shuttling protein exported from the nucleus to the cytoplasm dependent on CRM1 (chromosomal region maintenance 1). Moreover, its nucleocytoplasmic shuttling regulates synapsin I expression on the transcriptional level. Furthermore, synapsin I is decreased in sandy mice. Our data suggest that dysbindin transcriptionally regulates synapsin I gene expression dependent on its nuclear localization.
**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Full-length human dysbindin-1A cDNA was amplified, using a human adult brain cDNA library as the template, by PCR with primers 5′-GAAGATCTGT-CAATTGCTGGAGACCCCTTC-3′ and 5′-GGGATCCCATCT-CCAGCATAAGAGGAGACCCTTC-3′, and then inserted in frame into pET-21a (Novagen, Darmstadt, Germany) at BamHI/EcoRI sites. Recombinant dysbindin-1A cDNA (nucleotides 646–1056) from pGEX-5x-1 (Amersham Biosciences) and pDNA3.1/V5-HisA (Invitrogen) at BamHI/XhoI sites and inserted it into pEGFP-C2 (Clontech, Mountain View, CA) at BglII/EcoRI sites. pGEX-5x-1-dysbindin-1A and pcDNA3.1/V5-HisA-dysbindin-1A were constructed by subcloning the PCR products, amplified with primers 5′-GCGGATCCCATCT-CCAGCATAAGAGGAGACCCTTC-3′ and 5′-CCGCCTC- GAGTTAAGGTCGTTCGCCCTTC-3′, into pGEX-5x-1 (Amersham Biosciences) and pcDNA3.1/V5-HisA (Invitrogen) at BglII/PstI sites and then subcloned it into pEGFP-N3-dysbindin-1A at BglII/PstI sites and then subcloned it into pEGFP-N3 at EcoRI/BglII sites. It was then excised from human embryonic kidney 293 (HEK293) cells and inserted into BglII/EcoRI sites. pGEX-5x-1-dysbindin-1A and pcDNA3.1/V5-HisA-dysbindin-1A were constructed by subcloning the PCR products, amplified with primers 5′-GAAGGAAAGTGCCACCATG-GTAGC-3′ and 5′-TCTTTTTGGGGATGGATC-3′, into pET-21a (Novagen, Darmstadt, Germany) at BamHI/EcoRI sites or into pEGFP-N3 at EcoRI/BglII sites. It was then excised from human embryonic kidney 293 (HEK293) cells and inserted into pEGFP-N3 at EcoRI/BglII sites. It was then excised from human embryonic kidney 293 (HEK293) cells and inserted into pEGFP-N3 (Clontech, Mountain View, CA) at BglII/EcoRI sites. pGEX-5x-1-dysbindin-1A and pcDNA3.1/V5-HisA-dysbindin-1A were constructed by subcloning the PCR products, amplified with primers 5′-GAAGGAAAGTGCCACCATG-GTAGC-3′ and 5′-TCTTTTTGGGGATGGATC-3′, into pGEX-5x-1 (Amersham Biosciences) and pcDNA3.1/V5-HisA (Invitrogen) at BglII/XhoI sites and inserted it into pEGFP-C2 (Clontech) at BglII/PstI sites. Mutant dysbindin-1A Δ233–256 and dysbindin-1A Δ243A,I246A,L252A,L256A were generated using a site-directed mutagenesis kit (Takara, Otsu, Shiga, Japan) with primers 5′-A CCTCTGGAGGAAGAGC-3′ and 5′-TTCCATGATGACATGCTG-3′ for dysbindin-1A Δ233–256 and primers 5′-G GACGCATCGGACCAG-3′ and 5′-GAAGATCTACT-GAGCCAGCGGCCTC-CTGC-3′ for dysbindin-1A Δ243A,I246A,L252A,L256A. pEGFP-N3-NLS was constructed using a site-directed mutagenesis kit (Takara) with primers 5′-AGAGGAAAGTGCCACCATG-GTAGC-3′ and 5′-TCTTTTTGGGGATGGATC-3′. Then dysbindin-1A cDNA was inserted into pEGFP-N3-NLS via BglII and Sall sites. Human synapsin I promoter DNA (~302 bp plus 3 bp) was obtained by PCR using primers 5′-CGGAATTCATATA-TGGCATCCCTCTATCG-3′ and 5′-GAAGATCTACT-GAGCCAGCGGCCTAGTC-3′ with genomic DNA extracted from human embryonic kidney 293 (HEK293) cells and inserted into pEGFP-N3 at EcoRI/BglII sites. It was then excised from pEGFP-N3-synapsin I (~302 to +3) at Smal/BglII sites and inserted into pGL3-Basic vector (Promega, Madison, WI). The fidelity of all constructs was confirmed by sequencing.

**Protein Purification**—Dysbindin-1A His, which was expressed in Escherichia coli strain BL21 (DE3) transformed with pET-21a-dysbindin-1A, was purified using His-Bind resin (Novagen, Darmstadt, Germany) and eluted according to the manufacturer’s instructions. The purified dysbindin-1A His was dialyzed and concentrated using centrifugal filter devices (Millipore, Billerica, MA).

**Antibody Preparation**—Polyclonal antisera against dysbindin-1A were raised by immunizing New Zealand White rabbits or BALB/c mice with purified dysbindin-1A His protein as an antigen.

**GST Pull-down Assay**—An aliquot containing 20 μg of GST or GST-dysbindin-1A that was expressed in E. coli strain JM109 was incubated with 20 μl of Glutathione-Sepharose 4B (Amersham Biosciences) for 20 min at room temperature. Sepharose bound with GST or GST-dysbindin-1A was incubated with 50 μg of protein from HEK293 cell lysates for 1 h at 4 °C. After incubation, the beads were washed four times with 1 ml of HNTG buffer (20 mM Hepes-KOH, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, and 10% glycerol) to remove unbound proteins. Bound proteins were eluted from the beads by boiling in SDS sample buffer and subjected to immunoblot analysis.

**Cell Culture and Transfection**—HEK293 and Neuro2a (N2a) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen). Dissociated HF cultures were prepared from postnatal 1-day-old Sprague-Dawley rat HF. Cells were gently dissociated with a plastic pipette after digestion with 0.5% trypsin (Invitrogen) at 37 °C. The dissociated cells were plated at a final density of 5 × 10^5 cells/cm² on polyethyleneimine-coated 6-well plates (Corning Glass) and cultured in Neurobasal medium (Invitrogen) containing 1 × B27 supplement (Invitrogen) and 3 μg/ml glucose (Sigma). Three days after culture, 5-fluoro-2′-deoxyuridine and uridine (Sigma) were added to a final concentration of 10 μM to repress the growth of glial cells.

For transfection, cultured cells were washed with Opti-MEM and then transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM without serum. The same volume of DMEM containing 10% FBS or primary cell culture medium was added to the culture medium 6 h after transfection. Forty-eight hours after transfection, cells were observed using an inverted system microscope IX71 (Olympus, Tokyo, Japan) or subjected to immunoblot analyses or immunoprecipitation assays.

**Subcellular Fraction**—After being washed twice with pre-cold phosphate-buffered saline (PBS), N2a cells were lysed in fractionation buffer containing 320 mM sucrose, 3 mM CaCl₂, 2 mM MgAc, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 0.5% Nonidet P-40 for 20 min on ice. Following centrifugation at 600 × g for 10 min at 4 °C, the supernatant was collected as the cytoplasmic fraction. The pellet was washed once with the fractionation buffer without Nonidet P-40 and then lysed in nuclear lysis buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 280 mM KCl, 0.2 mM EDTA, 1 mM DTT, and 0.5 mM PMSF and 0.3% Nonidet P-40 as the nuclear fraction.

**Dysbindin-1A siRNA Knockdown**—Double-stranded oligonucleotides targeting positions 182–204 (5′-AAGUGA- CAAGUCAAGAGAAGAC-3′) of human dysbindin-1A mRNA were synthesized by Shanghai GenePharma (Shanghai,
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China). Meanwhile, a nonspecific control siRNA served as a negative control. The transfection was performed with Oligofectamine (Invitrogen) according to the manufacturer’s instructions.

**Immunoprecipitation**—HEK293 cells or N2a cells transfected with FLAG-tagged constructs were collected 48 h after transfection. The cells were sonicated in cell lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protein inhibitor mixture (Roche Applied Science). Cellular debris was removed by centrifugation at 12,000 × g for 15 min at 4°C. The supernatants were incubated with monoclonal anti-FLAG antibody (Sigma) or polyclonal anti-dysbindin-1A antisera for 1 h at 4°C. After incubation, protein G-agarose (Roche Applied Science) was used for precipitation. The beads were washed with cell lysis buffer four times, and then bound proteins were eluted with SDS sample buffer for immunoblot analysis.

**Immunoblot Analysis**—Proteins were separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membrane (Millipore). The following primary antibodies were used: monoclonal anti-FLAG or anti-FLAG-HPR antibody (Sigma), monoclonal anti-GAPDH antibody (Millipore), monoclonal anti-GFP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-CRM1 antibodies (Santa Cruz Biotechnology, Inc.), polyclonal anti-dysbindin-1A antisera, polyclonal anti-Max antibodies (Santa Cruz Biotechnology, Inc.), and polyclonal anti-synapsin I antibodies (Santa Cruz Biotechnology, Inc.). Sheep anti-mouse IgG-HRP antibody or anti-rabbit IgG-HRP antibody (Amer sham Biosciences) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc.) were used as the secondary antibody. The proteins were visualized using an ECL detection kit (Amersham Biosciences).

**Statistical Analysis**—Densitometric analysis of immunoblot assays from three independent experiments was performed using Adobe Photoshop CS 8.0 (Adobe, San Jose, CA). Data were analyzed using Origin 7.5 (OriginLab, Northampton, MA).

**Animal Experiments**—The sdy mutant (sdy/sdy) and wild type (+/+ ) mice were derived from heterozygotes that were obtained from the Jackson Laboratory (Bar Harbor, ME). To examine the genotypes of +/+, +/sdy, and sdy/sdy, we performed PCR to genotype the mice based on the nature of the deletion mutation in the DTNBPI gene (24). The cortex and HF of mice were homogenized in cell lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate with protein inhibitor mixture (Roche Applied Science). The lysates were subjected to immunoblot analysis. For semiquantitative RT-PCR, total RNA of the HF was isolated using an SV total RNA isolation system (Promega), and 0.1 µg of RNA from each preparation was used as the PCR template. RT-PCR was performed using a two-step RNA PCR kit (AMV) (Takara) with the following specific primer pairs: 5’- AATCCGGGCCAGTG-3’ and 5’- TGGTGTCGGTCGCCTCAG-3’ for synapsin I; 5’- GACCTGACAGACTACTCTC-3’ and 5’- GACAGTGGAGGGCGGAGGATG-3’ for β-actin; 5’- CTGGTGACAGCCGAGGTTG-3’ and 5’- CTCGCCTCTCTGCGATGTC-3’ for dysbindin-1A.

All mice were maintained in a specific pathogen-free environment, and all animal experiments were approved by the Animal Welfare Advisory Committee of the University of Science and Technology of China.

**Dual Luciferase Reporter Gene Assay**—N2a cells were seeded into 12-well plates (5 × 10^5 cells/well) and cultured for 20 h in DMEM plus 10% FBS before transfection. Dysbindin-1A-EGFP or EGFP alone was co-transfected with synapsin I promoter reporter plasmids (pGL3-synapsin I (−302 to +3)) into N2a cells using Lipofectamine 2000. In each transfection, cells were also co-transfected with Renilla luciferase reporter plasmids (Promega). Firefly and Renilla luciferase activity were assayed with the Dual Luciferase Reporter Assay System using a Veritas Microplate luminometer according to the manufacturer’s instructions (Promega). The absolute values of firefly lucifercence were normalized to those of Renilla, and the ratios are presented as mean ± S.E. with three transfection experiments.

**RESULTS**

**Subcellular Distribution of Dysbindin-1**—There are three isoforms of dysbindin-1, dysbindin-1A, -1B, and -1C. Among them, dysbindin-1A is the full-length isoform of dysbindin-1 (23). To explore the functions of dysbindin-1, we first analyzed the dysbindin-1A protein sequence using the PSORT II Prediction WWW Server, a computer program for the prediction of protein localization sites in cells. The results showed that dysbindin-1 is predicted to be localized to the nucleus with a probability of 47.8%. To examine if dysbindin-1 could be localized to the nucleus, we generated an EGFP-tagged dysbindin-1A construct and transfected it into HEK293 cells, N2a cells, and primary cultured neuronal cells from rat HF, respectively. Overexpressed dysbindin-1A-EGFP was mainly
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A. Subcellular localization of overexpressed dysbindin-1A-EGFP. HEK293 cells (a–c), N2a cells (d–f), and primary rat HF neuronal cultures (g–i) were transfected with dysbindin-1A-EGFP. The cells were visualized using a fluorescent microscope (magnification ×400). The nuclei were stained with Hoechst 33342 (blue; b, e, and h). Scale bars, 5 μm. B. Subcellular localization of endogenous dysbindin-1. HEK293 cells (a–c), N2a cells (d–f), and primary rat HF neuronal cultures (g–i) were stained with anti-dysbindin-1 antisera and visualized using a fluorescent microscope (magnification ×400). The nuclei were stained with DAPI (1 μg/ml) (blue; b, e, and h). Scale bars, 5 μm. C. Endogenous dysbindin-1A in N2a cells is distributed in both the cytoplasmic and the nuclear fractions. C, cytoplasmic fraction; N, nuclear fraction. GAPDH and Max were used here as specific cytoplasmic and nuclear markers. IB, immunoblot.

cytoplasmic in the above cells (Fig. 1A and supplemental Fig. S1), whereas EGFP alone was diffusely distributed in whole cells (data not shown). We next examined the subcellular localization of endogenous dysbindin-1 by immunocytochemical staining using anti-dysbindin-1 antisera. In HEK293 cells, N2a cells, and primary cultured neuronal cells from rat HF, dysbindin-1 was also localized primarily to the cytoplasm (Fig. 1B). Recently, it was reported that endogenous dysbindin-1 is distributed to both the nuclear and the cytoplasmic fractions of SH-SY5Y cells (49). We therefore performed subcellular fractionation analysis followed by immunoblot analysis. In N2a cells, endogenous dysbindin-1A was distributed in both the cytoplasmic and the nuclear fractions (Fig. 1C), suggesting that despite apparent cytoplasmic localization, dysbindin-1 is distributed in both the cytoplasm and the nucleus.

Translocation of Dysbindin-1 from the Cytoplasm to the Nucleus upon LMB Treatment—Dysbindin-1 is distributed in both the cytoplasm and the nucleus. However, it is apparently primarily localized to the cytoplasm. We speculated that dysbindin-1 might be a nucleocytoplasmic shuttling protein whose nuclear import and export are well regulated. Nuclear export of a protein is usually dependent on a leucine-rich nuclear export signal (NES); we therefore analyzed the potential NES(s) in dysbindin-1A using the NetNES 1.1 server. The results showed that there are three potential NESs within dysbindin-1A. In many cases, nuclear export is mediated by its NES binding to the export receptor CRM1 in a Ran-GTP-dependent manner (50). We therefore transfected dysbindin-1A-EGFP into HEK293 cells, N2a cells, and primary cultured neuronal cells from rat HF, respectively. The transfected cells were treated with leptomycin B (LMB), an inhibitor of CRM1-mediated nuclear export. In the above cells, dysbindin-1A-EGFP was relocated to the nucleus and distributed in both the nucleus and the cytoplasm after LMB treatment, whereas the distribution of EGFP alone was not changed (Fig. 2, A–C). In primary cultured neuronal cells from rat HF, endogenous dysbindin-1 was also relocated to the nucleus upon treatment with LMB (Fig. 2D). These data demonstrated that dysbindin-1 is a CRM1-dependent nucleocytoplasmic shuttling protein.

To further confirm that the nuclear export of dysbindin-1 is associated with CRM1, we performed in vitro GST pull-down assays. GST-dysbindin-1A interacted with endogenous CRM1 from HEK293 cell lysates, whereas GST alone did not (Fig. 2E). In HEK293 cells overexpressing FLAG-dysbindin-1A, endogenous CRM1 was co-immunoprecipitated when FLAG-dysbindin-1A was immunoprecipitated with anti-FLAG antibody (Fig. 2F). In N2a cells, endogenous CRM1 was also co-immunoprecipitated when endogenous dysbindin-1A was immunoprecipitated with anti-dysbindin-1A antisera but not with the nonspecific rabbit IgG (Fig. 2G). These results suggest that there are physical interactions between dysbindin-1 and CRM1 in cells.

Identification of a Functional Leucine-rich NES in Dysbindin-1—Of the three predicted NESs, two are located in the N terminus (aa 1–217), and the other is located in the C terminus (aa 217–216). To identify these potential NESs, we generated two deletion mutants of dysbindin-1A, EGFP-tagged N terminus (aa 1–217) and C terminus (aa 216–351) (Fig. 3A). We transfected N2a cells with full-length dysbindin-
FIGURE 2. Nucleocytoplasmic shuttling of dysbindin-1. A–C, enrichment of overexpressed dysbindin-1A-EGFP in the nucleus upon treatment with LMB. HEK293 cells (A), N2a cells (B), and primary rat HF neuronal cultures (C) were transfected with EGFP (a–c and g–i) or dysbindin-1A-EGFP (d–f and j–l) and treated with LMB (2 ng/ml) (g–l) or equal volumes of EtOH (a–f) for 1 h. The cells were visualized using a fluorescent microscope (magnification ×400). Scale bars, 5 μm in A and B and 10 μm in C. D, enrichment of endogenous dysbindin-1 in the nucleus upon treatment with LMB in primary rat HF neuronal cultures. The cells were treated with LMB (2 ng/ml) (e–h) or equal volumes of EtOH (a–d) for 1 h. The cells were then stained with anti-dysbindin-1 antisera and anti-Tuj1 antibody and visualized using a fluorescent microscope (magnification ×400). The nuclei were stained with DAPI (1 μg/ml) (blue). Scale bars, 5 μm. E, dysbindin-1A interacts with CRM1 in GST pull-down assays. F, dysbindin-1A interacts with CRM1 in transfected HEK293 cells. G, endogenous dysbindin-1A interacts with endogenous CRM1 in N2a cells. IB, immunoblot; IP, immunoprecipitation.
A, N terminus, and C terminus, respectively. The cells were treated with LMB or ethanol (EtOH), the solvent of LMB. In the presence of EtOH, the N terminus of dysbindin-1A was distributed throughout the cells, similar to EGFP; however, the C terminus of dysbindin-1A was mainly localized in the cytoplasm, similar to full-length dysbindin-1A (Fig. 3B). In the presence of LMB, most of the C terminus of dysbindin-1A was relocated to the nucleus, whereas the subcellular localization of the N terminus of dysbindin-1A was not affected (Fig. 3B). These data suggest that the NES may exist in the C terminus of dysbindin-1A. NES sequences that are recognized by CRM1 include four closely spaced hydrophobic residues, particularly leucine residues (51). The hydrophobic residues within NES are requisite for its nuclear export (52). We therefore generated a deletion mutant of dysbindin-1A to delete a leucine-rich NES-like region (aa 233–256) and a four-point mutant (L243A,I246A,L252A,L256A) to further characterize the NES (Fig. 3C). The cytoplasmic localization of dysbindin-1A was disrupted when the leucine-rich NES-like region (aa 233–256) was deleted, and it was distributed throughout the cells.

FIGURE 3. Identification of a functional leucine-rich NES in dysbindin-1. A, schematic illustration of different EGFP-tagged expression constructs. B, the C terminus of dysbindin-1A contains a LMB-sensitive NES. N2a cells were transfected with EGFP alone (a–f) or EGFP-tagged full-length dysbindin-1A (aa 1–351) (g–l), EGFP-tagged N terminus of dysbindin-1A (aa 1–217) (m–r), or EGFP-tagged C terminus of dysbindin-1A (aa 216–351) (s–x). Cells were treated with LMB (2 ng/ml) (d–f, j–l, p–r, and v–x) or equal volumes of EtOH (a–c, g–i, m–o, and s–u) for 1 h. Nuclei were stained with Hoechst 33342. Scale bars, 5 μm. C, schematic illustration of the potential NES (aa 233–256) of dysbindin-1A. D, 243LMDISDQEAALDVFL256 is a functional NES of dysbindin-1A. N2a cells were transfected with EGFP alone (a–c), EGFP-tagged dysbindin-1A (d–f), EGFP-tagged deletion mutant of dysbindin-1A (g–i), or dysbindin-1A with mutations in its potential NES (L243A,I246A,L252A,L256A) (j–l). Scale bars, 5 μm.
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A

FIGURE 4. Regulation of synapsin I expression by nucleocytoplasmic shuffling of dysbindin-1. A, overexpression of dysbindin-1A increases synapsin I expression in N2a cells. N2a cells were transfected with EGFP alone or EGFP-tagged dysbindin-1A. The cells were collected 48 h after transfection and subjected to immunoblot analyses with anti-GFP antibody, anti-synapsin I antibodies, or anti-GAPDH antibody. Band density of synapsin I relative to that of GAPDH was quantified to indicate the expression level of synapsin I. -Fold induction compared with wild type dysbindin-1A (supplemental Fig. S1) was shown as mean ± S.E. from three independent experiments. *, p < 0.05, one-way ANOVA. B, knockdown of endogenous dysbindin-1 decreases synapsin I expression. N2a cells were transfected with si-dysbindin-1A or a nonspecific control siRNA. The cells were collected 72 h after transfection and subjected to immunoblot analyses with anti-dysbindin-1A antisera, anti-synapsin I antibodies, or anti-GAPDH antibody. Band density of synapsin I or dysbindin-1A relative to that of GAPDH was quantified to indicate the expression level of synapsin I or dysbindin-1A. -Fold repression compared with the nonspecific control siRNA is shown as mean ± S.E. from three independent experiments. *, p < 0.05, one-way ANOVA. C, up-regulation of synapsin I expression upon treatment with LMB. N2a cells were treated with LMB (2 ng/ml) or equal volumes of EtOH for 3 h. Then the cells were collected and subjected to immunoblot analyses with anti-dysbindin-1A antisera, anti-synapsin I antibodies, or anti-GAPDH antibody. Band density of synapsin I relative to that of GAPDH was quantified to indicate the expression level of synapsin I. -Fold induction compared with EtOH control is shown as mean ± S.E. from three independent experiments. *, p < 0.05, one-way ANOVA. D, translocation of dysbindin-1A from the cytoplasm to nucleus upon treatment with LMB. N2a cells were treated with LMB (2 ng/ml) or equal volumes of EtOH for 1 h. Then the cells were subjected to subcellular fractionation and immunoblot analyses with anti-dysbindin-1A antisera, anti-GAPDH antibody, and anti-Max antibodies. Band density of dysbindin-1A relative to that of GAPDH or that of Max was quantified to indicate the expression level of dysbindin-1A in the cytoplasmic or nuclear fraction, respectively. -Fold repression or induction compared with EtOH control is shown as mean ± S.E. from three independent experiments. *, p < 0.05, one-way ANOVA. E, nuclear enrichment of dysbindin-1A promotes synapsin I expression. N2a cells were transfected with EGFP-tagged dysbindin-1A, NES mutant (mNES) dysbindin-1A (L243A,L246A,L252A,L256A), and dysbindin-1A-NLS, respectively. The synapsin I levels were higher in cells transfected with NES mutant dysbindin-1A or dysbindin-1A-NLS than those transfected with dysbindin-1A (Fig. 4E and supplemental Fig. S4). These data suggest that (Fig. 3D). Similar results were obtained using the four-point mutant (Fig. 3D). These results suggest that LMDIS-DQEALDVFL is a functional NES in dysbindin-1.

Up-regulation of Synapsin I by Translocation of Dysbindin-1 from the Cytoplasm to the Nucleus—It has been reported that overexpression of dysbindin-1 induces synapsin I expression and that knockdown of endogenous dysbindin-1 decreases synapsin I expression in rat primary cortical neuronal cultures (6). We also observed that overexpression of dysbindin-1A-EGFP up-regulated synapsin I expression (Fig. 4A) and that knockdown of endogenous dysbindin-1 decreased synapsin I expression (Fig. 4B) in N2a cells. We therefore examined whether nucleocytoplasmic shuttling of dysbindin-1 has effects on its regulation on synapsin I expression. As shown in Fig. 4C, expression of synapsin I was increased when N2a cells were treated with LMB. Using fractionation analysis combined with immunoblot analysis, we observed that endogenous dysbindin-1A was decreased in the cytoplasmic fraction and increased in the nuclear fraction in N2a cells treated with LMB (Fig. 4D). To further identify the effects of the nuclear dysbindin-1 on synapsin I expression, we generated a construct with an artificial SV40 T antigen nuclear localization signal (NLS) fused to dysbindin-1A-EGFP (dysbindin-1A-NLS-EGFP) (supplemental Fig. S2A). Dysbindin-1A-NLS-EGFP was distributed diffusely in both the cytoplasm and the nucleus in N2a cells (supplemental Fig. S2B). We next transfected N2a cells with EGFP-tagged dysbindin-1A, NES mutant dysbindin-1A (L243A,L246A,L252A,L256A), and dysbindin-1A-NLS, respectively. The synapsin I levels were higher in cells transfected with NES mutant dysbindin-1A or dysbindin-1A-NLS than those transfected with dysbindin-1A (Fig. 4E and supplemental Fig. S4). These data suggest that...
translocation of dysbindin-1 from the cytoplasm to the nucleus enhances synapsin I expression.

Decrease of Synapsin I in Sandy Mice—We next examined the synapsin I protein levels in the brains of sandy (sdy) mice, a dysbindin-1-null strain (24). In both cortex and HF, the synapsin I levels were lower in sandy mice than in wild type mice (Fig. 5A). Consistent with the data from immunoblot analyses, the synapsin I immunoreactivities were also lower in the HF in sandy mice than in wild type mice (Fig. 5B), and in the HF, the mRNA level of synapsin I was also decreased in sandy mice (Fig. 5C). We next tested if dysbindin-1 has effects on synapsin I transcription. To address this possibility, we constructed the promoter of the synapsin I gene (−302 to +3 bp) into pGL3 luciferase reporter vector and performed the reporter gene assays. In N2a cells, overexpression of dysbindin-1A-EGFP significantly activated the transcription of the luciferase reporter gene (Fig. 5D). These results suggest that dysbindin-1 regulates synapsin I expression in mice and that its regulation is at the transcriptional level.

DISCUSSION

Schizophrenia has a high heritability, and genetic factors are thought to have an important role in schizophrenia (53). In recent years, genetic studies have identified several candidate genes conferring risk for schizophrenia, including DISC1 (disrupted in schizophrenia 1) (54, 55), DTNBP1, NRG1 (neuregulin 1) (56, 57), DAOA (d-amino acid oxidase activator) (58), COMT (catechol-O-methyl transferase) (59), and others. Now more attention and effort are being devoted to the exploration of the roles of these genes and their products in schizophrenia (60, 61). Appropriate subcellular localization is important for a protein to fulfill its molecular function. In previous studies, it was reported that dysbindin-1 exerts its function mainly in the cytoplasm as a member of the BLOC-1, which regulates SNARE-mediated membrane fusion processes and has a role in neurite outgrowth (24, 34, 62, 63). In the brain, dysbindin-1 interacts with snapin, another member of the BLOC-1, and regulates its stabilization (28, 33, 37). Dysbindin-1 also modulates dopamine D2 receptor trafficking and signaling through BLOC-1 (31, 39).

Besides its cytoplasmic localization, dysbindin-1 is also reported to be localized to the nucleus (1, 20, 49, 64). It was found that endogenous dysbindin-1 in SH-SY5Y cells is distributed to both the cytoplasm and the nucleus by subcellular fractionation assay and interacts with nuclear DNA-dependent protein kinase complex, although it is primarily localized to the cytoplasm as detected by immunocytochemistry (49).

In our present study, we demonstrate that dysbindin-1 is a nucleocytoplasmic shuttling protein (Figs. 1 and 2). Consistent with these observations, endogenous dysbindin-1 is detected in both the cytoplasm and the nucleus (Fig. 1C). The preferentially cytoplasmic distribution of dysbindin-1 observed in Fig. 1, A and B, exhibits the steady state of the dynamic shuttling process and can be altered by inhibition of its nuclear export (Fig. 2). Nuclear import and export of proteins larger than ~40 kDa are highly regulated in eukaryotic cells. Usually, these proteins harbor NLS and NES, and they need importins and exportins to mediate their nuclear import and export.
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export (65). There is no classical NLS within dysbindin-1 detectable by analyzing the dysbindin-1A sequence using the PredictNLS server. However, we demonstrate that dysbindin-1 interacts with exportin-1/CRM1 and contains a functional leucine-rich NES (aa 243–256) (Figs. 2 and 3). The apparent cytoplasmic localization of dysbindin-1 was changed when the cells were treated with LMB, an inhibitor of CRM1-mediated nuclear export. It has also been reported that dysbindin-1 is localized to the nucleus in the HF of human brain (20). Taking these data together, they demonstrate that dysbindin-1 is a nucleocytoplasmic shuttling protein. Moreover, the subcellular distribution of the N terminus of dysbindin-1A is similar to the distribution of dysbindin-1A-NLS (Fig. 3B and supplemental Fig. S2B). These results imply that the N terminus of dysbindin-1A may harbor a “default” NLS. There may be a balance between the effect of the NLS and NES to determine the effective concentration of dysbindin-1 in the nucleus.

Consistent with the findings by other investigators, our data show that synapsin I is up-regulated by dysbindin-1 (Fig. 4). In the present study, we further show that translocation of dysbindin-1 from the cytoplasm to the nucleus significantly increases synapsin I expression (Fig. 4E). Moreover, we show that dysbindin-1 regulates synapsin I expression at the transcriptional level. Dysbindin-1 interacts with the synapsin I promoter (supplemental Fig. S3). The mRNA level of synapsin I was decreased in the HF of sandy mice (Fig. 5C), and the synapsin I promoter was transactivated by overexpression of dysbindin-1 in N2a cells (Fig. 5D). Interestingly, it was reported that full-length dysbindin-1 fused to the Gal4 DNA-binding domain is autoactivated in a Gal4-based yeast two-hybrid system (24, 33). Dysbindin interacts with a transcription factor, NF-YB, and regulates the transcriptional level of myristoylated alanine-rich protein kinase C substrate in the mouse brain (64). These results imply that dysbindin-1 may function in the nucleus to regulate gene transcription.

Synapsin I is a phosphoprotein specifically associated with the cytoplasmic surface of the synaptic vesicle membrane (66). It regulates the reserve pool of synaptic vesicles and the kinetics of neurotransmitter release (67). In the synapsin I-deficient mice, the size of the immediately releasable pool of vesicles is decreased, but the number of docked vesicles is unchanged (68, 69). Injection of synapsin domain E peptide, which disrupts the normal functions of endogenous synapsins, decreases the amount of transmitter release and slows the onset and decay of release, thus changing the release probability (70). In sandy mice, loss of dysbindin-1 affects the kinetics of transmitter release, and the size of the readily releasable pool is smaller but the number of docked vesicles is unchanged (71). These data demonstrate that a lack of dysbindin-1 results in changes similar to those caused by a decrease of synapsin I. Synapsin I- and synapsin II-null mice display an increased age-dependent cognitive impairment (72), and flies lacking all synapsins show impaired complex behavior (73). Studies on the post-mortem brain in schizophrenia patients have shown that synapsin I is decreased in HF of some schizophrenic brains (42, 46). Because dysbindin-1 is also decreased in HF in schizophrenia (20), this raises the possibility that synapsin I and dysbindin-1 are correlated in schizophrenia. In the present study, the correlation is evidenced in our in vivo observations that synapsin I is decreased in sandy mice (Fig. 5).

Our present study reveals that synapsin I expression is regulated by nucleocytoplasmic shuttling of dysbindin-1. The decrease of dysbindin-1 in the brain may lead to a decrease of synapsin I expression that consequently influences neurotransmission, thus playing roles in the pathogenesis of schizophrenia.

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