Mouse Disabled1 (DAB1) Is a Nucleocytoplasmic Shuttling Protein

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Disabled1 (DAB1) is an intracellular mediator of the Reelin-signaling pathway and essential for correct neuronal positioning during brain development. So far, DAB1 has been considered a cytoplasmic protein. Here, we show that DAB1 is subject to nucleocytoplasmic shuttling. In its steady state, DAB1 is mainly located in the cytoplasm. However, treatment with leptomycine B, a specific inhibitor of the CRM1 (chromosomal region maintenance 1)-RanGTP-dependent nuclear export, resulted in nuclear accumulation of DAB1. By using deletion or substitutional mutants of DAB1 fused with enhanced green fluorescent protein, we have mapped a bipartite nuclear localization signal and two CRM1-dependent nuclear export signals. These targeting signals were functional in both Neuro2A cells and primary cerebral cortical neurons. Using purified recombinant proteins, we have shown that CRM1 binds to DAB1 directly in a RanGTP-dependent manner. We also show that tyrosine phosphorylation of DAB1, which is indispensable for the layer formation of the brain, by Fyn tyrosine kinase or Reelin stimulation did not affect the subcellular localization of DAB1 in vitro. These results suggest that DAB1 is a nucleocytoplasmic shuttling protein and raise the possibility that DAB1 plays a role in the nucleus as well as in the cytoplasm.

The mammalian cerebral cortex is composed of six layers of various types of neurons having different birth dates, and the layer structure is essential for correct functioning of the central nervous system. This laminar construction is achieved by orchestrated migration of an appropriate number and type of neurons at appropriate time, because neurons are produced within places different from where they finally reside. The proper migration and positioning of neurons in the cerebral cortex depends on various signaling pathways (1–3). Among them, the Reelin-signaling pathway is significantly important for neuronal positioning in the developing brain (4, 5). Reelin is a large glycoprotein secreted by neuronal populations that occupy the most superficial layer of the brain, particularly Cajal-Retzius cells (6, 7). Mice with mutation in the reelin gene (reeler) show severe disruption of the laminar structure in the cerebral cortex, cerebellum, hippocampus, and several subcortical structures (8, 9).

Transmission of the Reelin signal to the migrating neurons requires the binding of Reelin to the very low density lipoprotein receptor and apolipoprotein E receptor 2 (10–12). Binding of Reelin to the receptors induces tyrosine phosphorylation of the cytoplasmic protein DAB1 (Disabled1) (12, 13). DAB1 contains a motif known as a protein interaction/phosphotyrosine-binding domain (14), which enables DAB1 to bind the cytoplasmic tail of both very low density lipoprotein receptor and apolipoprotein E receptor 2. dab1-deficient mice exhibit behavioral and anatomical abnormalities that are indistinguishable from those of reeler (15–18). Moreover, dab1 knock-in mice, which have point mutations in DAB1 so that they cannot be phosphorylated by Reelin stimulation, show a very similar phenotype to that of dab1-null mice (19). Therefore, it is thought that the tyrosine phosphorylation of DAB1 is essential for the activation of the downstream cascade of Reelin signaling.

In eukaryotic cells, the nuclear translocation of signal transduction proteins, such as transcription factors, is essential for cell processes, such as differentiation, transformation, and the control of gene expression (20, 21). Transport across the nuclear envelope occurs through the nuclear pore complex (NPC). Although NPC contains pathway for small molecules (smaller than 40–60 kDa or 9 nm in diameter) by diffusion (22), many cell proteins with nuclear function appear to be actively transported in and out of the nucleus through the NPC. Active nucleocytoplasmic transport is mostly mediated by a family of nuclear transport receptors belonging to the karyopherin family, also known as importins and exportins, by an energy-dependent mechanism (23, 24).

A protein destined for the nucleus or cytoplasm contains a specific sequence that can be recognized by importin or exportin.
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tin. The import sequence is provided by the nuclear localization signal (NLS), which interacts with the import receptor importin α and importin β complex (25–30). So far, two major types of NLSs have been identified for the nuclear import of proteins: classic NLS and bipartite NLS. The former was first found in the large T antigen of the SV40 virus and contains clusters of basic amino acids (31). The bipartite NLS was first identified in Xenopus nucleoplasmin and is composed of the following characteristic pattern: two basic residues, ~10–12-amino acid linker, and another basic region consisting of at least 3 basic residues of 5 residues (32). On the other hand, the export sequence is provided by a leucine-rich nuclear export signal (NES) (33, 34), which interacts directly with a class of exportins called exportin1 or CRM1 (chromosomal maintenance 1) in a RanGTP-dependent manner (35–39). Some proteins that contain both NLS and NES shuttle between the nucleus and the cytoplasm. The steady state localization of such proteins is regulated by the relative rates of their import and export. Extracellular signals influence these rates, so the extracellular signals can control the gene expression by regulating bidirectional movements of the signal transducer proteins (40).

In this study, we report that the DAB1 protein is able to shuttle between the cytoplasm and nucleus. In the presence of leptomycin B (LMB), an inhibitor of CRM1 (41), DAB1 accumulates in the nucleus. Mutagenesis analysis revealed that DAB1 contains two leucine-rich NES sequences and a bipartite NLS sequence. Furthermore, we demonstrated that DAB1 protein does indeed directly interact with CRM1 in a RanGTP-dependent manner.

EXPERIMENTAL PROCEDURES

Mice—The reeler mouse colony was originally derived from heterozygous B6C3Fe-a/a-rl adults (The Jackson Laboratory). Heterozygous reeler (+/rl) and homozygous reeler (rl/rl) mice were obtained by crossing rl/rl males with +/rl females. ICR mice were obtained from Japan SLC (Hamamatsu, Japan). The day on which a vaginal plug was detected was designated as embryonic day 0 (E0). All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals of the Keio University School of Medicine.

Plasmid Vectors—To construct the DAB1 expression vector (pCAGGS-DAB1), the EcoRI fragment of DAB1 was excised from the full-length mouse DAB1 cDNA construct pBluescript II (pBS)-mDab555 (14) (kindly provided by J. Cooper, Fred Hutchinson Cancer Research Center, Seattle, WA) and introduced into the EcoRI site of pCAGGS1, which has the modification in a multiple cloning site of a mammalian expression vector pCAGGS (42) (kindly provided by J. Miyazaki, Osaka University, Osaka, Japan).

To prepare the enhanced green fluorescence protein (EGFP) fusion protein expression vector, EGFP was amplified by PCR using specific primers EGFP-F3 (5′-gattctctggagagcggag-gagctc-3′) and EGFP-R (5′-gccgctgtctatcttgagctt-3′) with pEGFP-N1 (Clontech, Mountain View, CA) as a template. The amplified fragment was inserted into pGEM-T easy (Promega, Madison, WI). The EGFP fragment was excised by EcoRI/NotI digestion and inserted into the EcoRI/NotI site of pCAGGS1. The resulting plasmid pCAGGS1-EGFP/f was used to create EGFP fusion proteins.

An expression plasmid (pCAGGS-DAB1-EGFP) encoding full-length DAB1 and EGFP fusion proteins was constructed as follows. A DAB1 cDNA fragment was amplified by PCR using DAB1-8 (5′-ccgaatcagggagcggagaagacctgac-3′) and DAB1-9 (5′-ccctcctgacccttgagcttc-3′) with pBS-mDab555 as a template. The PCR fragment was cloned into the EcoRV site of pBS, generating pBS-DAB1/f. The DAB1 cDNA fragment was excised with EcoRI/Xhol digestion and inserted in frame with EGFP into the EcoRI/Xhol site of pCAGGS1-EGFP/f.

To generate a series of substitutional mutations in DAB1, site-directed mutagenesis was carried out as described by Zheng et al. (43). The NES1 of DAB1, 152-SDLNLQF160, was changed to 152-ADARDAAQA160. To make mutations L152A, L154A, and L160A, we first introduced mutations into Phe152, Leu154, and Leu160 using DAB1-152 (5′-atcgccggacgagagatcctgcggtcagcttgagcttc-3′) and DAB1-154 (5′-gcagctggagacgagagattcctgccggtcagcttgagcttc-3′), and the resulting plasmid was designated as pBS-DAB1NES1mt/f. NES2 of DAB1, 142-FDLISQNL469, was also changed to 142-ADASQANA160. To make mutations F462A, I464A, and L469A, we first introduced mutations into Lys462, Leu464, and Leu469 using DAB1-462 (5′-ctgactccgctgcttgtgttgtgctttcttggcgctgg-3′) and DAB1-464 (5′-agctgctgctgcttgtgttgtgctttcttggcgctgg-3′) using specific primers EGFP-F3 (5′-gattctctggagagcggag-gagctc-3′) and EGFP-R (5′-gccgctgtctatcttgagctt-3′) with pEGFP-N1 (Clontech, Mountain View, CA) as a template. The amplified fragment was inserted into pGEM-T easy (Promega, Madison, WI). The EGFP fragment was excised by EcoRI/NotI digestion and inserted into the EcoRI/NotI site of pCAGGS1.
DAB1NLSmt was excised by EcoRI/XhoI digestion and introduced into the EcoRI/XhoI site of pCAGGS-EGFP/f to generate pCAGGS-DAB1NLSmt-EGFP.

An expression plasmid (pCAGGS-DAB1NES1/2mtΔN-EGFP) encoding DAB1 with N-terminal deletion and substitutional mutation in both NES1 and NES2 was constructed as follows. First, the sequence corresponding to amino acids 37–555 of DAB1NES1/2mt was amplified by PCR using specific primers DAB1-23 (5’-ggtgctgcagcgaagcttttttaggcgctgggctgttttgatg-3’) and DAB1-24 (5’-gttggctgggcggcggctgggctgttttgatg-3’) with pBS-DAB1NES1/2mt/f as a template. The PCR product was inserted into the EcoRV site of pBS, followed by digestion with EcoRI/XhoI. The DAB1 N-terminal deletion mutant cDNA was inserted into the EcoRI/XhoI site of pCAGGS1-EGFP/f in frame with EGFP.

An expression contract (pCAGGS-DAB1NLSmtNES1/2mt-EGFP) encoding a fusion protein between EGFP and full-length DAB1 containing site-directed mutations in NLS, NES1, and NES2 was constructed as follows. A ~1-kb Apal fragment containing DAB1 N-terminal cDNA derived from pCAGGS-DAB1NLSmt-EGFP was ligated into a ~6-kb Apal fragment containing DAB1 C-terminal cDNA derived from pCAGGS-DAB1NES1/2mt-EGFP.

For the construction of the fusion protein between C-terminal deletion mutants of DAB1 and EGFP, the coding sequence of DAB1 proteins was amplified by PCR with pBS-mDb2555 or pBS-DAB1NES1mt/f as a template. We used the following combinations of primers to produce cDNA fragments encoding DAB1 NES1mt, NES2mt, and NES1mt/2mt combinations of primers to produce cDNA fragments encoding DAB1 proteins was amplified by PCR with pBS-mDab555 or pBS-DAB1NES1mt-EGFP as a template. We used the following primers to produce cDNA fragments encoding DAB1 NES2mt and subcloned into the EcoRV site of the pBS vector. The DAB1NES2 mt cDNA fragment was excised with EcoRI/NcoI digestion and inserted in frame with GST into the EcoRI/XhoI site. Likewise, the DAB1 NLS fragment with mutations in the basic amino acids (DAB1NLSmt; AAAGQDRSEATLIKRFK), which was created by annealing the two oligonucleotides 5’-aattcatgagatatgtgctgaccgtctcagctgtgtttgg-3’ and 5’-tgcctgataattcgtgctggtgctg-3’, was inserted into the EcoRI/XhoI site. The resulting plasmids were designated pGEX4T1-DAB1NLSmt-EGFP and pGEX4T1-DAB1NLSmt-EGFP, respectively.

The GST-DAB1NES1/2mt expression vector (pGEX4T1-DAB1NES1/2mt) was created as follows. First, a DAB1NES2mt cDNA fragment was amplified by PCR using DAB1-8 and DAB1-28 (5’-ggtgctgcagcgaagcttttttaggcgctgggctgttttgatg-3’) with pBS-DAB1NES2mt and subcloned into the EcoRV site of the pBS vector. The DAB1NES2 mt cDNA fragment was excised with EcoRI/NcoI digestion and inserted in frame with GST into the EcoRI/XhoI site. Likewise, the DAB1 NLS fragment with mutations in the basic amino acids (DAB1NLSmt; AAAGQDRSEATLIKRFK), which was created by annealing the two oligonucleotides 5’-aattcatgagatatgtgctgaccgtctcagctgtgtttgg-3’ and 5’-tgcctgataattcgtgctggtgctg-3’, was inserted into the EcoRI/XhoI site. The resulting plasmids were designated pGEX4T1-DAB1NLSmt-EGFP and pGEX4T1-DAB1NLSmt-EGFP, respectively.

The GST-DAB1NES1/2mt expression vector was constructed as follows. A DAB1-EGFP fragment was excised with EcoRI/NotI from pCAGGS-DAB1-EGFP and introduced into pGEX-4T-1 (GE Healthcare) to generate pGEX4T1-DAB1-EGFP. The cDNA fragment of DAB1 was removed with EcoRI/XhoI, and an oligonucleotide fragment encoding DAB1 NLS (KKQDQRTSEATLIAAF), which was created by annealing the two oligonucleotides 5’-aattcatgagatatgtgctgaccgtctcagctgtgtttgg-3’ and 5’-tgcctgataattcgtgctggtgctg-3’, was ligated into the EcoRI/XhoI site. The resulting plasmids were designated pGEX4T1-DAB1NLSmt-EGFP and pGEX4T1-DAB1NLSmt-EGFP, respectively.

The GST-DAB1NES1mt with mutations was then inserted into the EcoRI/XhoI site. The full-length Reelin expression vector pCrl (47) was kindly provided by T. Curran (Sr. Jude Children’s Research Hospital, Memphis, TN).

Culture and Transfection of Neuro2a Cells—For the subcellular localization studies of DAB1 or the various DAB1-EGFP fusion proteins, Neuro2a cells were grown at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 mg/ml streptomycin, and 100 units/ml penicillin. Transient transfection of plasmid DNA into Neuro2a cells was performed using Superfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. For experiments requiring inhibition of CRM1-dependent nuclear export, after 36 h in culture, transfected cells were treated with LMB (Sigma) at 10 ng/ml or the same volume of methanol as a vehicle control and incubated for another 1, 2, 3, 4, or 12 h.

Ex Utero Electroporation—To introduce plasmid DNAs into cerebral cortical neurons, we carried out an ex utero electroporation using E14.5 ICR mice as described by Tabata and Nakajima (48, 49) with modifications for the ex utero experiment. Dissected brains were immersed in phosphate-buffered saline

DAB1NES1mt, with EGFP to make pCAGGS-DAB1NES1mt-EGFP.

The GST-DAB1NES1mt and GST-DAB1NES2mt-EGFP expression vectors were constructed as follows. A DAB1-EGFP fragment was excised with EcoRI/NotI from pCAGGS-DAB1-EGFP and introduced into pGEX-4T-1 (GE Healthcare) to generate pGEX4T1-DAB1-EGFP. The cDNA fragment of DAB1 was removed with EcoRI/XhoI, and an oligonucleotide fragment encoding DAB1 NLS (KKQDQRTSEATLIAAF), which was created by annealing the two oligonucleotides 5’-aattcatgagatatgtgctgaccgtctcagctgtgtttgg-3’ and 5’-tgcctgataattcgtgctggtgctg-3’, was ligated into the EcoRI/XhoI site. The resulting plasmids were designated pGEX4T1-DAB1NLSmt-EGFP and pGEX4T1-DAB1NLSmt-EGFP, respectively.

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(138 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.2 mM KH₂PO₄), and plasmid DNA was injected into both lateral ventricles with a glass micropipette. The brain was placed between tweezer-type electrodes (CUY650P5; Nepa Gene Co., Chiba, Japan). Electric pulses (30 V, 50 ms) were charged eight times at 950-ms intervals for each hemisphere of the brain using a square pulse electroporator (CUY21EDIT; Nepa Gene Co.). After electroporation, brains were used for primary culture.

Primary Culture of Cerebral Cortical Neurons—Primary cerebral cortical neurons were prepared from mouse brains as described by Goslin and Banker (50) with a slight modification at E14.5 for the ex utero electroporation experiment and for observation of endogenous DAB1 subcellular localization or at E16.5 for the Reelin stimulation experiment. The dorsolateral regions of the cerebral cortices were dissected out, the meninges were stripped away, and the isolated brain tissues were then treated with a trypsin (2.5%) and DNase (80 μg/ml) solution for 15 min at 37 °C. After washing three times with calcium- and magnesium-free Hanks’ balanced salt solution, the brain tissues were triturated by pipetting to dissociate the cells. The cells were then plated at a cell density of 1 × 10⁵ cells/cm² on 6-cm plastic culture dishes for the Reelin stimulation experiment. The neurons were cultured in a neurobasal medium supplemented with B27 (Invitrogen), 0.5 mM l-glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin. For LMB treatment, after 36 h in culture, cells were treated with methylamine (vehicle control) or 10 ng/ml LMB for another 12 h.

Reelin-containing Medium and Reelin Stimulation—293T cells on a 10-cm culture dish were transfected with pcDNA encoding full-length Reelin cDNA with Superfect transfection reagent. After 3 h of transfection, the transfection reagent was replaced with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 mg/ml streptomycin, and 100 units/ml penicillin. Two days later, mock- and Reelin-containing supernatants were collected and stored at 4 °C until use. The mock- or Reelin-containing medium was applied to mouse embryonic cortical neurons at 3 days in culture, and the neurons were stimulated for 10, 30, and 60 min.

Immunoprecipitation—For immunoprecipitation, primary neurons on 6-cm culture dishes or Neuro2a cells on 35-mm culture dishes were collected, lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0% Nonidet P-40, 5 mM EDTA, 50 mM NaF, and 1 mM Na₃VO₄) containing a protease inhibitor mixture (Roche Applied Science), and clarified by centrifugation. The clarified lysates were reacted with a rabbit anti-DAB1 antibody (Chemicon International, Temecula, CA) overnight at 4 °C on a rotator, and then protein G beads were added and incubated for an additional 1 h. The beads were precipitated, washed three times with lysis buffer, eluted by boiling in SDS sample buffer, and analyzed by 7.5% SDS-PAGE. After electroblotting onto a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA), tyrosine phosphorylation was detected with a horseradish peroxidase-conjugated pY20 monoclonal antibody (BD Transduction Laboratories, San Jose, CA). The blot was stripped with SDS and reprobed with the mouse anti-DAB1 monoclonal antibody D4, which was kindly provided by A. Goffinet (University of Louvain, Brussels, Belgium).

Immunocytochemistry—For the immunocytochemical analysis, Neuro2a cells or primary neurons were fixed with 4% paraformaldehyde in PBS for 10 min, washed, and permeabilized with an extraction solution (PBS containing 5% bovine serum albumin and 1% Triton X-100) for 15 min. The cells were then incubated with primary antibodies against rabbit anti-DAB1 antibody, mouse monoclonal anti-Fyn antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-histone antibody (Chemicon International), or mouse monoclonal anti-class III β-tubulin antibody (TuJ1; Babco, Richmond, CA) for 60 min, washed, and further incubated for 60 min with the goat anti-rabbit IgG TRITC (Jackson Immunoresearch Laboratories, West Grove, PA), goat anti-mouse IgG fluorescein isothiocyanate (Jackson Immunoresearch Laboratories), and TO-PRO-3 iodide (Molecular Probes, Inc., Eugene, OR). For detection of endogenous DAB1 in the primary neurons, goat biotin-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was applied as the secondary antibody and visualized by reaction with fluorescein isothiocyanate-conjugated streptavidin (Vector Laboratories).

Criteria for Subcellular Distribution of Proteins—The following criteria were used to determine the subcellular distribution of DAB1 and DAB1-EGFP fusion proteins. For the cytoplasmic localization (C), the protein signal is more intense in the cytoplasm than the nucleus. For the nuclear localization (N), the protein signal is more intense in the nucleus than the cytoplasm. The cytoplasmic and nuclear localization (C + N) protein signal is equally distributed in the cytoplasm and nucleus.

Subcellular Fractionation and Western Blot Analysis—Cerebral cortices were dissected out from E16.5 +/rl or rl/rl mouse brains and stored at −80 °C until use. After genotyping as described previously (51), the +/rl or rl/rl cerebral cortices were homogenized in 100 μl of homogenization buffer (0.32 M sucrose, 25 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, Tris-HCl (pH 7.4), and protease inhibitor mixture) and centrifuged at 2300 × g for 10 min. The supernatant was stored as a cytosolic fraction. The pellet was resuspended in 500 μl of homogenization buffer and centrifuged at 2300 × g for 10 min. This step was repeated two times. The final nuclear pellet was resuspended in 100 μl of SDS-sample buffer and sonicated for 10 0.8-s sonications using a probe type sonicator (TOMY UD-201; Tomy, Tokyo, Japan). The nuclear and cytoplasmic fractions of each sample (50 μg) were then separated by 7.5% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with antibodies against monoclonal anti-mouse DAB1 antibody (D4). The blot was stripped with SDS and reprobed with an anti-histone antibody or a goat anti-actin antibody (Santa Cruz Biotechnology) to show the purity of fractionation.

Microinjection—Neuro2a cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and plated on coverslips 12–18 h before use. Recombinant GST-DAB1NLS-EGFP (3.7 mg/ml) or GST-DAB1NLsmt-EGFP (3.3 mg/ml) was injected through a glass capillary into the cells on coverslips using a semi-automated microinjection system (Eppendorf, Hamburg, Germany). Where indicated, 2 mg/ml wheat germ agglutinin. 
(WGA) was injected. After incubation for 2 h at 37 or 4 °C, the cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The cells were washed with PBS, and immunocytochemistry was carried out as described above.

Expression and Purification of Recombinant Proteins—To express GST and GST fusion proteins, Escherichia coli strain BL21 codon plus (Stratagene, La Jolla, CA), which had been transformed with pGEX4T1, pGEX4T3-DAB1 (kindly provided by L. Keshvara, The Ohio State University, Columbus, OH), pGEX4T1-DAB1NES1/2mt, pGEX4T1-DAB1NLS-EGFP, or pGEX4T1-DAB1NLsmt-EGFP, was grown in LB medium containing 50 μg/ml ampicillin at 27 °C to a density of 0.5 to 0.7. Expression was induced by the addition of 2 mM isopropyl-β-D-thiogalactopyranoside and incubation for 5 h at 27 °C. Cells were harvested by centrifugation, and the pellets were frozen and stored at −80 °C until use. The pellets were resuspended in lysis buffer (PBS containing 1 mM EDTA, protease inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride) and sonicated with BIORUPTOR, an ultrasonic cell rupturing device (Cosmo Bio, Tokyo, Japan). The lysates were centrifuged at 4 °C for 20 min at 15,000 rpm, and the supernatants were then stored at −80 °C.

To obtain recombinant zz (two immunoglobulin G-binding domains of protein A)- and His-tagged human CRM1 (zz-CRM1-His6, hereafter referred to as zz-CRM1) and His-tagged human Ran (His10-RanQ69L, hereafter referred to as RanQ69L), we used pQE70-zz-CRM1 (52) (kindly provided by L. W. Mattaj, EMBL, Heidelberg, Germany) and pQE32-RanQ69L (53) (kindly provided by D. Gorlich, ZMBH, University of Heidelberg, Heidelberg, Germany), and the recombinant proteins were prepared as described by Askjaer et al. (54) with some modifications. The E. coli strain M15[pREP4] (Qiagen, Valencia, CA) was transformed by the plasmids and cultured in LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin at 27 °C to a density of 0.5 to 0.7. The recombinant proteins were induced by 2 mM isopropyl-β-D-thiogalactopyranoside at 27 °C for 5 h. After centrifugation, the cell pellets were washed with PBS, and the proteins were then stored at −80 °C.

Cytoplasmic Localization of DAB1 Is Mediated by CRM1-dependent Nuclear Export—During analysis of the primary structure of the DAB1 protein, we noticed that DAB1 (NCBI Protein accession: CAA69662) contains a bipartite NLS (32) at amino acid positions 20–36 (20RKKGQDRSEATLIKRFK36) using PSORT II on the PSORT World Wide Web server. We therefore attempted to verify whether this NLS motif could indeed transport DAB1 from the cytoplasm to the nucleus. To trace the subcellular localization of DAB1 in living cells, we constructed a DAB1-EGFP fusion protein expression vector, transiently expressed it in mouse neuroblastoma Neuro2a cells, and observed the distribution at 2 days after transfection. Contrary to our expectation, however, DAB1-EGFP protein was exclusively distributed throughout the cytoplasm, and almost no signals were observed in the nucleus (data not shown). This observation suggested that subcellular localization of DAB1 was somehow limited to the cytoplasm, although it contains an NLS sequence. It is currently recognized that a number of nuclear proteins possess NES in addition to NLS, and the localization of the proteins can be tightly regulated through the modulation of both nuclear import by NLS and export by NES (20, 21, 40). The NES sequence binds to the nuclear export receptor CRM1/Exportin1 (35–39), and the interaction NES-CRM1 can be blocked by a fungal antibiotic, LMB (35, 41, 56), resulting in an inhibition of NES-dependent protein export from the nucleus. To test whether the LMB influenced the distribution of DAB1-EGFP, we transiently introduced the DAB1-EGFP expression plasmid into Neuro2a cells. At 36 h after transfection, the cells were treated with 10 ng/ml LMB (or an equivalent volume of methanol as a vehicle control) for 12 h. In the absence of LMB, almost 100% of the DAB1-EGFP-transfected cells showed cytoplasmic distribution (Fig. 1, A (top) and B). However, LMB treatment induced accumulation of DAB1-EGFP fusion protein within the nuclei in 71% of the transfected cells and led to equal distribution between the cytoplasm and nucleus in 29% of
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DAB1 Contains Two Nuclear Export Signals—To provide insights into the molecular mechanisms of nucleocytoplasmic shuttling of DAB1, we searched for the presence of NES sequence within DAB1 using an NES prediction server, NetNES1.1 (available on the World Wide Web at www.cbs.dtu.dk/services/NetNES/) (57). As a result, it was revealed that nine residues, from leucine 152 to leucine 160 (152LDLRDLFQL160), have an NES motif score of more than 0.5. This program also showed that eight residues, from phenylalanine 462 to leucine 469 (462FDISQNLN469), have a hidden Malcof model score of more than 0.060. Furthermore, since the consensus for the NES motif is quite loose (57), we searched another NES motif using the loose NES consensus, (L/I/V/F/M)X2,3(L/I/V/F/M)X2,3(L/I/V/F/M)X2,3(L/I/V/F/M)X2,3(L/I/V/F/M)X2,3(L/I/V/F/M). We detected the following five sequences matching this motif: 87LTLISFGGIK106, 150VILDLRDLFQL160, 193VEPDVYQYIVE203, 240YSAVTQLFEL248, and 442ESSYFNKGYG452. The putative NES motif 152LDLRDLFQL160 predicted by NetNES1.1 was also contained in these sequences. Thus, we finally predicted six putative NES-like motifs within the DAB1 primary sequence and tentatively designated them as PNES1 to PNES6, respectively (Fig. 2A).

To determine which putative NES-like sequence is indeed functional, we constructed plasmid vectors expressing fusion proteins of EGFP and a series of DAB1 C-terminal deletion mutants (Fig. 2A). These plasmid vectors were transiently transfected into Neuro2a cells, and their subcellular localization was observed (Fig. 2B and C). As a result, these EGFP fusion proteins showed a quite different distribution from each other. The DAB1-EGFP and DAB1ΔC1-EGFP fusion proteins localized exclusively in the cytoplasm, whereas DAB1ΔC2-EGFP, DAB1ΔC3-EGFP, DAB1ΔC4-EGFP, and DAB1ΔC5-EGFP fusion proteins localized evenly in both the nucleus and cytoplasm. Furthermore, DAB1ΔC6-EGFP and DAB1ΔC7-EGFP proteins localized exclusively in the nucleus. These results suggest that amino acid residues 148–183 contain a nuclear export activity as predicted by the NES score of NetNES1.1 or the consensus motif search, and also N-terminal 80 amino acid residues contain nuclear import activity as predicted by PSORT II. Moreover, since the cytoplasmic localization of DAB1-EGFP was changed to even distribution by C-terminal deletion of DAB1 in DAB1ΔC2-EGFP, it is also suggested that the C-terminal region (amino acid residues 460–478) contains another nuclear export activity, as predicted by the hidden Malcof model score of NetNES1.1. Thus, we designated the PNES2 as NES1 and the PNES6 as NES2.

Previous studies suggested that hydrophobic residues, especially leucine, within the leucine-rich NES are critical for its nuclear export activity (33, 34). To determine whether the NES1 sequence within amino acid residues 148–183 is required for the nuclear export of DAB1 and to further confirm the presence of NES2 between amino acids 459 and 478, we introduced five point mutations into hydrophobic residues of NES1 (152LDLRDLFQL160) with alanine (L152A, L154A, L158A, F158A, and L160A) and constructed an expression vector to express the fusion protein of EGFP and full-length protein or five C-terminal deletion mutants (Fig. 3A). The expression vectors were transiently transfected into Neuro2a cells, and the distribution of DAB1-EGFP fusion proteins was compared the transfected cells (Fig. 1, A (bottom) and B). Similar results were obtained when a wild-type DAB1, which had not been fused with EGFP, was expressed and treated with LMB (Fig. 1, C and D). To determine how long the transfected Neuro2a cells would have to be treated with LMB to achieve DAB1-EGFP redistribution, we next treated the transfected Neuro2a cells with LMB for 1–4 h. Nuclear accumulation of DAB1-EGFP was observed even at 1 h after the LMB treatment (Fig. 1, E and F, and data not shown). Notably, the proportions of cells showing a nuclear or an equal distribution, between the cytoplasm and nucleus, were similar to those after 12-h treatment with LMB. This suggested that the relatively long treatment (12 h) did not secondarily affect the subcellular redistribution of DAB1, at least not severely. These results indicate that DAB1 protein is subject to shuttling between the nucleus and the cytoplasm and that the nuclear export may be mediated by an LMB-sensitive CRM1-dependent pathway.

FIGURE 1. Subcellular localization and nucleocytoplasmic shuttling of DAB1-EGFP and DAB1 in Neuro2a cells. A, C, and E, DAB1-EGFP (A and E) or DAB1 (C) was transiently expressed in Neuro2a cells, and 36 h after transfection, the cells were treated with methanol (vehicle control) (upper panel in A and C) or 10 ng/ml LMB (E and lower panel in A and C). After 1 (E, lower panel) or 12 h (A and C) of treatment, cells were fixed, permeabilized, and stained with an anti-histone antibody (right row in A, C, and E) to visualize the nucleus. Wild-type DAB1 was stained with rabbit anti-DAB1 antibody. The position of nucleus is indicated by white dotted lines. B, D, and F, quantitative analysis of the distribution of DAB1-EGFP (B and F) or DAB1 (D) in transfected cells. More than 100 transfected cells were observed for each expression plasmid and classified into three different categories as described under “Experimental Procedures”: C (black bar), cytoplasmic distribution; C = N (gray bar), equal distribution between cytoplasmic and nuclear compartment; N (white bar), nuclear distribution. Scale bars in A, C, and E represent 10 μm.
with the subcellular localization of the DAB1-EGFP deletion mutants without substitutional mutations in the NES1 (Fig. 2). As shown in Fig. 3, B and C, DAB1NES1mt-EGFP and DAB1NES1mtΔC1-EGFP were still found mainly in the cytoplasm. On the other hand, introduction of mutations into NES1 resulted in redistribution of mutant DAB1NES1mtΔC2-EGFP, DAB1NES1mtΔC3-EGFP, DAB1NES1mtΔC4-EGFP, and DAB1NES1mtΔC5-EGFP fusion proteins almost exclusively to the nucleus. These results confirmed that NES1 is a functional NES and that the C-terminal region (amino acid residues 460–478) contains nuclear export activity, as predicted in Fig. 2.

As shown in Fig. 1, DAB1 was suggested to be subject to CRM1-mediated nuclear export, and this export was inhibited by LMB. To investigate whether DAB1 nuclear export is dependent on NES1, NES2, or both NESs, we transiently expressed DAB1-EGFP, DAB1NES1mt-EGFP, or DAB1NES2mt-EGFP in Neuro2a cells and then treated them with methanol (vehicle control) or LMB (Fig. 4). For the DAB1NES2mt-EGFP construct, we substituted the hydrophobic residues of NES2 (462FDISQLNL469) to alanines (F462A, I464A, L467A, L469A) and constructed an expression vector of the EGFP fusion protein (Fig. 4A). In the absence of LMB, 100% of cells expressing DAB1-EGFP and 90% of cells expressing DAB1NES1mt-EGFP fusion proteins showed cytoplasmic distribution, respectively. On the other hand, the majority of cells (94%) expressing DAB1NES2mt-EGFP showed even distribution throughout the cell (Fig. 4B), suggesting that NES2 is more effectively contributing to the normal cytoplasmic distribution of wild-type DAB1 than NES1. In the presence of LMB, 75% of cells expressing DAB1-EGFP and 90% of cells expressing DAB1NES1mt-EGFP fusion proteins showed cytoplasmic distribution, respectively. On the other hand, the majority of cells (94%) expressing DAB1NES2mt-EGFP showed even distribution throughout the cell (Fig. 4B), suggesting that NES2 is more effectively contributing to the normal cytoplasmic distribution of wild-type DAB1 than NES1. In the presence of LMB, 75% of cells expressing DAB1-EGFP showed predominant localization in the nucleus, and the rest of the cells (25%) showed equal distribution, in a similar manner to the results in Fig. 1, A and B. When the CRM1-dependent protein export was inhibited by LMB, the DAB1NES1mt-EGFP fusion protein was distributed to the

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**FIGURE 2. Identification of NES in DAB1.** A, schematic representation of DAB1-EGFP fusion proteins. Six putative NES-like sequences (PNES1 to -6) in DAB1 are indicated in blue, one putative NLS sequence (NLS) is shown in red, and the phosphotyrosine-binding domain (PTB) is shown in orange. The numbers indicate the amino acid positions of the part of the DAB1 protein included in the fusion protein. As described under “Experimental Procedures,” full-length DAB1 cDNA or truncated DAB1 cDNA fragments without the stop codon were subcloned in frame at the 5'-end of the sequence encoding EGFP. B, subcellular localization of indicated various DAB1-EGFP fusion proteins (green) in Neuro2a cells. Expression vectors of DAB1-EGFP fusion proteins were transiently transfected into Neuro2a cells. After 2 days in vitro, the cells were fixed, permeabilized, and stained with an anti-histone antibody (magenta). C, quantitative analysis of the subcellular localization pattern of each fusion protein. More than 100 transfected cells were observed for each expression plasmid and classified into three different categories (C, C = N, and N as described in the legend to Fig. 1). Scale bar, 10 μm.
nucleus in 91% of the transfected cells. In the case of DAB1NES2mt-EGFP, LMB treatment resulted in nuclear accumulation in 61% of the cells and even distribution in the rest of the cells.

To confirm whether the nuclear export activity of DAB1 is mainly dependent on the NES1 and NES2, we transiently expressed DAB1NES1/2mt-EGFP fusion protein, which has substitutional mutations in both NES1 and NES2 (Fig. 4A), in Neuro2a cells. As a result, DAB1NES1/2mt-EGFP was almost completely localized in the nuclei (100%) even in the absence of LMB (Fig. 4, B and C). LMB treatment had no effect on the nuclear accumulation of DAB1NES1/2mt-EGFP. These observations clearly showed that the nuclear export activity of DAB1 is mainly dependent on both NESs. These results suggest that DAB1 shuttles between the nucleus and the cytoplasm and that the two NESs are responsible for the nuclear export.

RanGTP-dependent Binding of CRM1 to DAB1—As mentioned above, CRM1 binds to NES-containing cargo proteins cooperatively with RanGTP. The NES-Cargo-CRM1-RanGTP ternary complex is translocated through the NPC toward the cytoplasm without GTP hydrolysis (35, 36, 39). If CRM1 functions as an export receptor for DAB1, it must bind DAB1 directly in a RanGTP-dependent manner. To determine whether CRM1 does indeed bind to DAB1 in a RanGTP-dependent manner, we used an in vitro CRM1 binding assay in the presence of RanQ69L GTP. RanQ69L cannot hydrolyze GTP and thus remains in an active GTP-bound state. GST-tagged DAB1 was immobilized on glutathione Sepharose beads, and zz-CRM1 was added in the presence or absence of RanQ69L GTP. The beads were extensively washed, and the presence of bound zz-CRM1 was determined by immunoblot analysis. Binding of zz-CRM1 to GST-DAB1 was markedly increased in the presence of RanQ69L GTP (Fig. 5, top, lane 4). On the other hand, no obvious binding of zz-CRM1 to GST alone was observed (Fig. 5, top, lanes 1 and 2).

Moreover, we determined whether the NES sequences in DAB1 were required for CRM1 binding. To this end, we prepared a GST-DAB1 fusion protein with point mutations in phenylalanines or leucines of both NESs (GST-DAB1NES1/2mt). As a result, the
interaction between DAB1 and CRM1 was significantly reduced, and virtually no signal was observed (Fig. 5, top, lane 6). Taken together, our results suggest that CRM1 directly binds to DAB1 through the NESs in a RanGTP-dependent manner and that DAB1 is actively exported to the cytoplasm by CRM1.

DAB1 Contains a Nuclear Localization Signal—The PSORT II program predicted that DAB1 contained a bipartite NLS in the N terminus of DAB1. In fact, as shown in Fig. 2, B and C, DAB1NES1/2mt-EGFP, which is composed of 80 N-terminal amino acids of DAB1 and EGFP, constitutively localized to the nucleus. To test whether the predicted NLS sequence indeed contributed to DAB1 nuclear import, we first constructed an N-terminal deletion mutant of DAB1NES1/2mt-EGFP (Fig. 6A, DAB1NES1/2mt/H9004N-EGFP), and its localization pattern was assessed (Fig. 6, B (middle) and C). Although DAB1NES1/2mt-EGFP localized almost exclusively to the nucleus (97%), DAB1NES1/2mtΔN-EGFP mainly localized to the cytoplasm (87%). These results are consistent with the hypothesis that the N terminus containing amino acid residues 20–36 contributes to the DAB1 nuclear import. To test this further, the basic amino acids consisting of the bipartite NLS (20RKKGQDRSEATLIKRFK36) were converted to alanine (R20A, K21A, K22A, K33A, R34A, and K36A) to create DAB1NLSmtNES1/2mt-EGFP, and localization of this fusion protein was examined. As shown in Fig. 6, B (bottom) and C, almost all of the cells expressing DAB1NLSmtNES1/2mt-EGFP showed cytoplasmic distribution (95%). These results suggest that amino acid residues 20–36 could function as a bipartite NLS signal. However, these experiments by themselves cannot exclude the alternative possibility that the fusion proteins diffused via the nuclear pores and thus might have been retained in the nucleoplasm by binding to other nuclear proteins.

To exclude this possibility, we determined whether the NLS of

FIGURE 4. Mutational analyses of NES1 and NES2. A, the NES2 sequence of DAB1 is given in the single-letter code at the top. Neutral alanine was introduced into the hydrophobic residues indicated by the red color. A schematic representation shows DAB1-EGFP fusion proteins having substitutional mutation in NES1, NES2, or both NESs. The putative NLS is shown in red, and the two NESs are shown in blue. The phosphotyrosine-binding domain is the orange region. The numbers above the scheme indicate the amino acid position of DAB1. ×, substitutional mutation into NESs. B, subcellular localization of DAB1-EGFP fusion proteins. DAB1-EGFP fusion proteins (green) were transiently transfected into Neuro2a cells. Thirty-six hours after transfection, cells were treated with methanol (vehicle control) or 10 ng/ml LMB for 12 h, and then cells were fixed, permeabilized, and stained with an anti-histone antibody (magenta). C, quantitative analysis of the subcellular distribution of fusion proteins. More than 100 transfected cells were observed for each expression plasmid and classified into three different categories (C, C = N, and N). Scale bar, 10 μm.
Nucleocytoplasmic Shuttling of DAB1

DAB1 has the capacity to migrate into the nucleus. We created a chimeric protein, GST-DAB1NLS-EGFP, consisting of GST fused with DAB1 bipartite NLS (RKKGQDRSEATLIKRFK) and EGFP (Fig. 6D) and microinjected it into the cytoplasm of Neuro2a cells. At 2 h after microinjection, the subcellular localization of EGFP signal was examined. As shown in Fig. 6E (top), GST-DAB1NLS-EGFP accumulated in the nucleus. To rule out the possibility that GST-DAB1NLS-EGFP had entered the nucleus through passive diffusion, we first examined the temperature dependence of GST-DAB1NLS-EGFP nuclear import. GST-DAB1NLS-EGFP was injected into the Neuro2a cytoplasm, and the cells were incubated at 4 °C. No accumulation of GST-DAB1NLS-EGFP was observed in the nucleus (Fig. 6E, middle), indicating that GST-DAB1NLS-EGFP was imported by an active temperature-sensitive mechanism. We further examined whether WGA inhibited the nuclear import of GST-DAB1NLS-EGFP. WGA is known to block nuclear transport of molecules through the NPC by binding to the O-linked GlcNAc residues of glycoproteins in the nuclear membrane (58–60). This effect of WGA does not block the passive diffusion of small molecules. As shown in Fig. 6E (bottom), GST-DAB1NLS-EGFP migration was blocked by coinjection of WGA (2 mg/ml). To further confirm the nuclear import activity of DAB1 NLS, substitutional mutations were introduced into the basic amino acid clusters of DAB1 bipartite NLS (Fig. 6D), and the resulting mutant protein, GST-DAB1NLSmt-EGFP, was microinjected into the Neuro2a cytoplasm (Fig. 6F). These mutations significantly affected the nuclear import activity of GST fusion protein, and virtually no signal was observed in the nucleus. Taken together, our results suggest that the NLS of DAB1 is functional and has nuclear import activity.

Both NLS and NES Sequences of DAB1 Are Functional in Primary Cerebral Cortical Neurons—To investigate whether the DAB1-EGFP fusion protein expressed in primary neurons was also subject to nucleocytoplasmic shuttling, we then examined the localization of DAB1-EGFP in the absence or presence of LMB in dissociated cerebral cortical neurons (Fig. 7, A and B). We first introduced the DAB1-EGFP expression vector into cells within the ventricular zone of the dorsolateral cerebral cortex using ex utero electroporation at E14.5. The electroporated areas of the cerebral cortex were dissected out immediately after the electroporation, dissociated into single cells, and cultured on glass coverslips. At 36 h after the electroporation, methanol (vehicle control) or LMB was added to the medium and incubated for another 12 h. The cells were stained with an anti-class III β-tubulin (TuJ1) antibody to identify neurons. In the absence of LMB, dominant distribution of DAB1-EGFP was observed in the cytoplasm (68%), whereas the treatment with LMB caused nuclear distribution in 29% of TuJ1-positive neurons. These results suggested that DAB1-EGFP was also subjected to nucleocytoplasmic shuttling in the primary cerebral cortical neurons, although the proportion of cells showing nuclear accumulation of DAB1-EGFP in the primary neurons was lower than that in Neuro2a cells.

In Neuro2a cells, the NLS and NES sequences of DAB1 contribute to its nucleocytoplasmic shuttling. To investigate whether the NLS and NES sequences of DAB1 are also responsible for the nucleocytoplasmic shuttling of DAB1-EGFP in primary neurons, we transiently expressed expression vectors for DAB1-EGFP, DAB1NES1/2mt-EGFP, or DAB1NLSmtNES1/2mt-EGFP into cells in the ventricular zone of the cerebral cortex at E14.5 and cultured the cells on glass coverslips. After 2 days in vitro, distribution of the DAB1-EGFP fusion proteins in TuJ1-positive neurons was examined (Fig. 7, C and D). As a result, differing from the observation of Neuro2a cells (Fig. 1, A and B), only 69% of neurons expressing DAB1-EGFP showed cytoplasmic-dominant distribution, and the rest of the neurons (31%) showed uniform distribution throughout the cell body and the nucleus. DAB1NES1/2mt-EGFP accumulated in the nucleus in all neurons (100%), whereas DAB1NLSmtNES1/2mt-EGFP exclusively localized to the cytoplasm of the primary neurons (98%). These results indicated that both the NLS and NES sequences were also functional in primary neurons and contributed to DAB1 nucleocytoplasmic shuttling.

Nucleocytoplasmic Shuttling of Endogenous DAB1—We then investigated whether the nucleocytoplasmic shuttling that we observed for the exogenously transfected DAB1 or DAB1-EGFP fusion protein reflects the behavior of endogenous DAB1 proteins. For this purpose, cerebral cortical cells were obtained from E14.5 mice and cultured on coverslips. After 36 h of incubation, methanol (vehicle control) or LMB was added to the medium, and the cells were incubated for another 12 h. The subcellular localization of endogenous DAB1 in TuJ1-positive neurons was determined with an anti-DAB1 antibody (Fig. 7E). In the absence of LMB, endogenous DAB1 was detected mainly in the cytoplasm as punctate signals, although a faint signal of DAB1 might also be detected in the nucleus. When the cells were incubated with LMB, the accumulation of endogenous DAB1 to the nucleus was observed. From these results, we concluded that DAB1 is a nucleocytoplasmic shuttling protein in primary cerebral cortical neurons.
Subcellular Distribution of DAB1 Is Not Affected by Tyrosine Phosphorylation by Fyn—Although tyrosine phosphorylation of DAB1 is known to be especially required for the Reelin-DAB1 signaling pathway, its exact physiological meaning remains unclear. Since the most common regulatory mechanism of the nucleocytoplasmic transport of proteins is phosphorylation/dephosphorylation reactions (20, 21), we hypothesized that DAB1 phosphorylation might affect the DAB1 subcellular localization. To date, it has been believed that the tyrosine kinase that phosphorylates DAB1 would be an Src family kinase, such as Src and Fyn, because an inhibitor of Src family kinase prevented phosphorylation of DAB1 in primary neurons (61, 62) and caused a reeler-like malformation in cortical brain slices (63). Additionally, Src and Fyn double mutant mice showed a reeler-like phenotype (64). Therefore, we next examined whether the subcellular localization of DAB1 was affected by Fyn-dependent tyrosine phosphorylation. Expression vectors of Fyn WT, Fyn KD, or Fyn CA were cotransfected with the DAB1-EGFP expression vector into Neuro2a cells. DAB1-EGFP proteins were immunoprecipitated with an anti-DAB1 antibody and immunoblotted with either a DAB1 antibody to reveal the total amount of DAB1 protein levels or with an anti-phosphotyrosine antibody, pY20, to display the amount of tyrosine-phosphorylated DAB1. The results revealed that the DAB1-EGFP fusion protein was efficiently tyrosine-phosphorylated when coexpressed with Fyn WT or Fyn CA but was not or was only slightly tyrosine-phosphorylated when expressed alone or coexpressed with Fyn KD, respectively (Fig. 8A). However, subcellular localization of DAB1 was not obviously affected by the degree of tyrosine phosphorylation (Fig. 8B).

Reelin Stimulation Does Not Influence the Subcellular Distribution of Endogenous DAB1 in Primary Cerebral Cortical Neurons—Tyrosine phosphorylation of DAB1 is known to be induced by adding Reelin-containing medium to embryonic forebrain neurons in culture (13). We therefore tested whether tyrosine phosphorylation of DAB1 triggered by Reelin stimulation would affect the subcellular localization of endogenous DAB1 in vitro. E16.5 mouse cerebral cortices were dissected out, dissociated, and cultured. After 3 days in culture, the neurons were treated with a mock-transfected control medium or Reelin-containing medium for 10, 30, or 60 min. After the treatment, DAB1 tyrosine phosphorylation and subcellular distribution was observed (Fig. 9, A and B). DAB1 was phosphorylated by Reelin treatment even at 10 min after the stimulation. Nonetheless, the subcellular distribution of endogenous DAB1 was not changed by Reelin treatment at each time point.

DAB1 Exists in both Nuclear and Cytoplasmic Fractions of +/rl and rl/rl Mouse Brains—To investigate whether the presence of Reelin affects the subcellular localization of DAB1 in vivo, the heterozygous (+/rl) or homozygous reeler (rl/rl)
mouse cerebral cortices were biochemically fractionated into nuclear and cytoplasmic fractions, and the subcellular localization of endogenous DAB1 was investigated (Fig. 9C). Immunoblotting of these fractions indicated that DAB1 was present in both nuclear and cytoplasmic fractions not only in +/H11001/+H11001 mouse brains but also in rl/rl mouse brains. Furthermore, as previously reported (65), the amount of DAB1 in the cytoplasmic fraction was higher in the rl/rl mice compared with the +/H11001/+H11001 mice. Although an increase of DAB1 protein in the nucleus was also observed in the rl/rl mouse, the distribution ratio in the cytoplasm and the nucleus was not dramatically changed in the mutant.

**DISCUSSION**

**DAB1 Is a Nucleocytoplasmic Shuttling Protein**—In this study, we report that DAB1 is a nucleocytoplasmic shuttling protein. Nucleocytoplasmic shuttling of DAB1 was clearly shown by the treatment with LMB, an inhibitor of CRM1-dependent nuclear export. After treatment with LMB, DAB1...
the cytoplasm after treatment with LMB (Fig. 7B). Additionally, cytoplasmic retention of DAB1 after LMB treatment was also observed in endogenous DAB1 in primary cerebral cortical neurons (Fig. 7E). It is plausible that exogenous DAB1-EGFP and endogenous DAB1 in primary neurons may be anchored to certain molecules in the cytoplasmic region, including phospholipids of the plasma membrane (66, 67) and transmembrane proteins, such as apolipoprotein E receptor 2, very low density lipoprotein receptor (10), APLP1 (68), Pcdh18 (69), and other DAB1-interacting cytoplasmic proteins that have so far been reported: N-WASP (70), Nckβ (71), and Crk family proteins (72–74). Thus, the partial effect of LMB on primary neurons may in part have been due to a relatively small fraction of DAB1 in a free form being translocated to the nucleus.

Because DAB1 and DAB1-EGFP have an apparent molecular mass of ~80 and ~100 kDa, respectively, nucleocytoplasmic shuttling by passive diffusion is unlikely. It is known that small molecules can passively diffuse through NPCs via a concentration gradient, whereas macromolecules larger than 40–60 kDa cannot enter the nucleus through size limitation and must be actively transported across the NPCs. Thus, active nucleocytoplasmic shuttling mechanisms are likely to exist for the transportation of DAB1 proteins across NPCs. In this study, we revealed that DAB1 contained at least one NLS and two NESs (Figs. 2–4 and 6). Substitutional mutations in both NES1 and NES2 resulted in complete accumulation of DAB1-EGFP fusion protein in the nucleus of Neuro2a cells (Fig. 4, B and C) and primary cerebral cortical neurons (Fig. 7, C and D). Therefore, we conclude that nuclear export of DAB1 is mainly achieved by the use of these two NESs. Although both NES1 and NES2 are capable of mediating the nuclear export of DAB1-EGFP fusion proteins, substitutional mutation analysis suggested that the NES2 mediated stronger nuclear export activity than the NES1 (Fig. 4, B and C). We also showed that CRM1 bound directly to DAB1 in a RanGTP-dependent manner through the NES sequences (Fig. 5), indicating that nuclear export of DAB1 is mediated by CRM1. On the other hand, NLS has been mapped in the N-terminal region of DAB1 (amino acid residues 20–36) (Figs. 2 and 6). Nuclear import assay confirmed that NLS of DAB1 has the capacity to migrate into the nucleus. Collectively, our results indicate that the nucleocytoplasmic shuttling of DAB1 is regulated by both an NLS-dependent nuclear import mechanism and NESs-dependent nuclear export mechanism, although we are not attempting to rule out other additional and as yet unknown mechanisms.

Tyrosine Phosphorylation of DAB1 Does Not Affect Its Subcellular Distribution—One of the most common mechanisms involved in the regulation of the nucleocytoplasmic transport of proteins is the phosphorylation/dephosphorylation reaction (20, 21). For example, dephosphorylation of a transcription factor NF-ATc by the Ca2+-dependent protein phosphatase calcineurin induces nuclear accumulation of NF-ATc (75). On the other hand, phosphorylation of NF-ATc by GSK3 (glycogen synthase kinase-3) causes nuclear export of NF-ATc (76). As described above, DAB1 tyrosine phosphorylation is an essential step that regulates neuronal positions (19). Therefore, we tested whether DAB1 tyrosine phosphorylation by Fyn tyrosine kinase or Reelin stimulation would affect the DAB1 subcellular local-

FIGURE 9. Reelin does not affect subcellular distribution of DAB1. A and B, cerebral cortical cells prepared from single littersmate embryos at E14.5 were dissociated, and the neurons were plated on 6-cm plastic dishes for analysis of DAB1 tyrosine phosphorylation or on glass coverslips for observation of endogenous DAB1 subcellular distribution. After 3 days, the mouse embryonic neurons were treated with mock- or Reelin-containing medium for 10, 30, or 60 min. After the treatment, immunoprecipitation (IP) or immunocytochemistry was performed. A, after the stimulation, endogenous DAB1 was immunoprecipitated using a rabbit anti-DAB1 antibody. The immunoprecipitates were resolved by 7.5% SDS-PAGE and immunoblotted (WB), and tyrosine phosphorylation was detected with an anti-phosphotyrosine antibody, pY20 (pY). After stripping, the membrane was reprobed with a mouse anti-DAB1 antibody (D4). B, after incubation with the conditioned medium, neurons were fixed, permeabilized, and stained with a rabbit anti-DAB1 antibody (green), an anti-class III β-tubulin antibody (TuJ1; magenta), and TO-PRO-3 iodide (blue). Scale bar, 5 μm. C, cerebral cortices derived from E16.5+/rl or rl/rl mice were lysed, and the lysates were fractionated into cytoplasmic (C) and nuclear (N) fractions as described under “Experimental Procedures.” An equivalent amount (50 μg) of the fractions was resolved on 10% SDS-PAGE and immunoblotted with a mouse anti-DAB1 antibody. The immunoblots were stripped and reprobed with an anti-actin antibody as a cytoplasmic marker and an anti-histone antibody as a nuclear marker.

became localized to the nucleus of Neuro2a cells (Fig. 1) and primary neurons (Fig. 7, A, B, and E), although the efficacy of LMB was different between Neuro2a cells and primary neurons. As shown in Fig. 7, A and B, exogenously expressed DAB1-EGFP in primary neurons accumulated in the nucleus to a lesser extent than DAB1-EGFP did in Neuro2a cells (Fig. 1, A and B), and a considerable proportion of primary neurons expressing DAB1-EGFP showed the retention of the protein in
ization (Figs. 8 and 9, A and B). However, the DAB1-EGFP fusion protein remained in the cytoplasm after tyrosine phosphorylation. Thus, the nucleocytoplasmic shuttling of DAB1 is likely to be independent of DAB1 tyrosine phosphorylation. We cannot exclude the possibility, however, that a small fraction of DAB1 may actually be affected by phosphorylation induced by Fyn or Reelin stimulation.

What is the Physiological Meaning of DAB1 Nucleocytoplasmic Shuttling?—What would happen if the distribution of DAB1 were changed? We have shown that a DAB1 C-terminal deletion mutant DAB1ΔC3-EGFP, which was composed of 271 N-terminal amino acids of DAB1 and EGFP, resulted in equal distribution between the cytoplasm and nucleus in Neuro2a cells (Fig. 2). Previously, Herrick and Cooper (77) produced a knock-in mutant mouse of dab1p45 expressing only a natural splice form of DAB1 p45 (aa 1–271, which has the same length as DAB1ΔC3). Although DAB1 p45 protein lacks the C-terminal region of DAB1, dab1p45/p45 homozygotes showed normal brain development. Thus, if DAB1 p45 protein is localized equally in both the nucleus and the cytoplasm in the dab1p45/p45 mice, the distribution ratio of DAB1 between the nucleus and cytoplasm per se may not matter for the basic Reelin-DAB1 signaling pathway. Although there is no direct evidence to suggest that nucleocytoplasmic shuttling of DAB1 is required for the Reelin-signaling pathway, our results do not exclude the possibility that a Reelin signal might eventually be transmitted to the nucleus via nucleocytoplasmic shuttling of DAB1.

In summary, although it seems clear that DAB1 has a role in the cytoplasm as a part of signaling complexes, the present study suggests that it may also have a function in the nucleus. The various phenotypes observed in the DAB1-deficient mice (15–17) may thus partly reflect the defect in the role of DAB1 in the nucleus. Further study is required to determine the DAB1 functions in the cytoplasm and the nucleus and to clarify the physiological significance of the nucleocytoplasmic shuttling of DAB1 protein.

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