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The LIS1-related NUDF Protein of *Aspergillus nidulans* Interacts with the Coiled-Coil Domain of the NUDE/RO11 Protein

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Abstract. The *nudF* gene of the filamentous fungus *Aspergillus nidulans* acts in the cytoplasmic dynein/dynactin pathway and is required for distribution of nuclei. NUDF protein, the product of the *nudF* gene, displays 42% sequence identity with the human protein LIS1 required for neuronal migration. Haploinsufficiency of the LIS1 gene causes a malformation of the human brain known as lissencephaly. We screened for multicopy suppressors of a mutation in the *nudF* gene. The product of the *nudE* gene isolated in the screen, NUDE, is a homologue of the nuclear distribution protein RO11 of *Neurospora crassa*. The highly conserved NH_2-terminal coiled-coil domain of the NUDE protein suffices for protein function when overexpressed. A similar coiled-coil domain is present in several putative human proteins and in the mitotic phosphoprotein 43 (MP43) of *X. laevis*. NUDF protein interacts with the *Aspergillus* NUDE coiled-coil in a yeast two-hybrid system, while human LIS1 interacts with the human homologue of the NUDE/RO11 coiled-coil and also the *Xenopus* MP43 coiled-coil. In addition, NUDF coprecipitates with an epitope-tagged NUDE. The fact that NUDF and LIS1 interact with the same protein domain strengthens the notion that these two proteins are functionally related.

Key words: dynein • dynactin • lissencephaly • nudF • LIS1

Introduction

During growth of the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*, nuclei migrate into the germtube and distribute evenly along the cell length (reviewed in Fischer, 1999; Xiang and Morris, 1999). Analysis of mutations that affect nuclear distribution in these two fungi and in *S. cerevisiae* has identified microtubules and cytoplasmic dynein and dynactin complexes as the main components of the nuclear distribution machinery (Oakley and Morris, 1980; Plamann et al., 1994; Xiang et al., 1994; Tinsley et al., 1996; Geiser et al., 1997). Other proteins whose functions are less well understood are also required for nuclear migration. One such protein, NUDF of *A. nidulans* (Xiang et al., 1995a), is particularly interesting because it is 42% identical to LIS1, a human protein whose haploinsufficiency causes a devastating brain malformation known as lissencephaly (smooth brain; Reiner et al., 1993; Hattori et al., 1994). Lissencephaly is characterized by a smooth cerebral cortex, which is thought to be the result of impaired neuronal migration to the cortex during brain development (Hirotsune et al., 1998; reviewed in Walsh, 1999; Walsh and Goffinet, 2000).

Genetic analysis indicates that *nudF* and its *S. cerevisiae* homologue *PAC1* function in the dynein/dynactin pathway (Xiang et al., 1995a; Geiser et al., 1997; Willins et al., 1997). Particularly intriguing is that mutations in the cytoplasmic dynein heavy chain (CDHC)[1] can suppress *nudF* mutations (Willins et al., 1997). However, none of the proteins identified so far as components of the purified dynein or dynactin complexes from animal sources appear to be homologues of NUDF/Pac1p or LIS1, and the role of NUDF/Pac1p protein in dynein/dynactin function is unclear. Recent studies of a LIS1 homologue in *Drosophila* (Liu et al., 1999; Swan et al., 1999) revealed that LIS1 acts as a cortical anchor for dynein during *Drosophila* oogenesis (Swan et al., 1999).

Additional evidence that the NUDF and LIS1 proteins are functionally related comes from their interaction with NUDC. The *nudF* gene was cloned fortuitously as an extra copy suppressor of the *nudC3* mutation in the *nudC* gene (Xiang et al., 1995a). Remarkably, a mammalian homologue of the NUDC protein, which is known to be functional in *A. nidulans* (Morris et al., 1997), binds LIS1 pro-

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[1]*Abbreviations used in this paper*: CDHC, cytoplasmic dynein heavy chain, GFP, green fluorescent protein; *nud*, nuclear distribution defective; ts, temperature sensitive; VSV-G, Vesicular Stomatitis Virus Glycoprotein.

tein (Morris et al., 1998b). Unfortunately, besides the detrimental effect of the *A. nidulans nudC3* mutation on the NUDF protein level, little is known about the function of the *nudC* gene. In *Aspergillus*, the *nudC* null mutant is phenotypically very different from *nudF* or CDHC null mutants (Chiu et al., 1997), and thus *nudC* does not necessarily function only in the dynein/dynactin pathway. Other proteins that have been reported to bind LIS1 include platelet-activating factor acetylhydrolase (Hattori et al., 1994), pleckstrin homology domains (Wang et al., 1995), p72syk-like tyrosine kinase (Brunati et al., 1996), and tubulin and microtubules (Sapir et al., 1997). It is not known if fungal homologues of LIS1 are involved in similar interactions.

Here we identify *nudE*, the *A. nidulans* homologue of the *N. crassa* nuclear distribution gene *ro-11*, as a multicopy suppressor of a mutation in the *nudF* gene. According to genetic data, the RO11 protein functions in the cytoplasmic dynein pathway, but its precise role is not clear (Minke et al., 1999). We show that *A. nidulans* NUDE and NUDF proteins interact in a two-hybrid system and in *A. nidulans* protein extracts and demonstrate the generality of this interaction by showing that a similar interaction occurs between the human NUDE/RO11 homologue and human LIS1.

Materials and Methods

Strains and Plasmids

A. nidulans strains used were 20.3.10 (*argB2; pyrG89; pabaA1; fwA1*), GR5 (*pyrG89; pyroA4; wA2*) (made by G. May), AO1 (*nudC3; pyrG89; pabaA1; nicA2; wA2*) (Osmani et al., 1990), SF2-9 (*ΔnudE::argB; argB2; pyrG89; pabaA1; fwA1*) (this work), XX3 (*nudA1; pyrG89; chaA1*) (Xiang et al., 1994), XX20 (*nudF6; pyrG89; yA2*), XX21 (*nudF7; pyrG89; yA2*) (Xiang et al., 1995a), XX21x6-17 (*nudE::(VSV-G)$_6$::pyrG; nudF7; pyrG89; yA2*) (this work), ΔF54 (*ΔnudF::pyr4; pyrG89; wA2; pyroA4*) (Willins et al., 1995). Construction of the pAid vector has been described by Xiang et al. (1999). pMS12 was obtained from the Fungal Genetics Stock Center. *X. laevis* MP3 cDNA clone (data are available from GenBank/EMBL/DDBJ under accession number U95097) was kindly provided by Todd Stukenberg (Harvard Medical School, Boston, MA). A human *LIS1* cDNA clone (accession number L13385) was obtained from Takeo Kubota (NHGRI, NIH, Bethesda, MD). A human EST clone with the accession number AA424443 was purchased from Genome Systems, Inc.

Cloning of the nudE Gene

The screen for *nudF7* multicopy suppressors was based on the method previously used by us to clone *A. nidulans* genes (Efimov and Morris, 1998). Genomic DNA fragments (5–20 kb) from the XX20 strain were ligated to the pAid vector, which is the *A. nidulans* replicating vector pHELP1 (Gems and Clutterbuck, 1993) carrying the *pyrG* gene as a selective marker. XX21 protoplasts were transformed with the ligation mixture and grown at 43°C on twenty-eight 10-cm plates in YAG (0.5% yeast extract, 2% glucose, 2% agar, and trace elements) with 0.6 M KCl. The total number of *pyrG*$^+$ transformants was $>3 \times 10^4$. 79 putative suppressor clones were identified as patches of yellow color in the gray background of unconidiating mycelium. In 71 of those, conidiation was mitotically unstable and was lost in the absence of selection for the *pyrG* gene. Plasmids from nine unstable transformants were rescued in *E. coli*. Seven plasmids contained different but overlapping inserts with the *nudF6* gene. Two plasmids contained overlapping inserts with a gene that displayed homology to the *N. crassa* gene *ro-11*, which we named *nudE*. A 4.8-kb fragment of *A. nidulans* genomic DNA from the smallest suppressor plasmid (~7-kb insert) was subcloned and sequenced. The position of the *nudE* gene was predicted using homology between its ORF and the RO11 protein. The presence of a single intron, as well as the positions of the initiator and terminator codons, were confirmed by PCR analysis and sequencing of an *A. nidulans* cDNA library. The *nudE* sequence has been deposited in the GenBank (accession no. AF085679).

Disruption and Epitope-tagging of the nudE Gene

To disrupt the *nudE* gene, the *A. nidulans argB* gene was inserted as a 1.7-kb EcoRI-BamHI fragment from pMS12 between the unique MfeI and BamHI sites of the *nudE* gene on a plasmid. This resulted in deletion of the first 1,233 bp of the *nudE* gene and 265 bp upstream of the *nudE* start. The disrupted gene was introduced into the wild-type *nudE* locus of the 20.3.10 strain by homologous double recombination with a linear DNA fragment. The correct integration was confirmed by Southern hybridization for two independent *argB*$^+$ transformants, which were designated SF2-3 and SF2-9.

Tagging of the NUDE protein with six copies of the epitope YTDIEMNRLGK, which is recognized by the monoclonal antibody P5D4, was done as follows. First, the plasmid pP5D4x6-3 was constructed. This carries the epitope-encoding sequence GTAGATCT [TAC ACC GAC ATC GAG ATG AAC CGC CTC GGC AAG (GGATCT)]$_5$ (TAC ACC GAC ATC GAG ATG AAC CGC CTC GGC AAG) GGATCCA flanked by NcoI sites in the pGEM-5Z vector. Second, plasmid pSH-1 was made by subcloning the NruI-AccI *A. nidulans* genomic fragment (2,621 bp), which contains the truncated *nudE* gene (residues 45–586 of the NUDE protein), at the EcoRV-ClaI sites of the pXX1 vector (Xiang et al., 1995b). The epitope-encoding NcoI-NcoI fragment from pP5D4x6-3 was inserted at the unique NcoI site of the pSH-1 in frame with the *nudE* gene to produce plasmid pSHx6-3. The XX21 strain was transformed with the circular pSHx6-3 plasmid and *pyrG*$^+$ transformants were analyzed by PCR. Four transformants, designated XX21x6-#8, 12, 15, 17, were found that had undergone a single recombination event at the *nudE* locus resulting in the insertion of the epitope sequence in the intact *nudE* gene.

Domain Analysis of the NUDE Protein

The constructs used for the experiments shown in Fig. 1 B all carry the same 4.8-kb region of the *A. nidulans* genomic DNA at the SmaI site of pAid. The *nudE* gene is approximately in the middle of the insert and is oriented toward the AMA1 insert. Changes within the *nudE* gene were made as follows. The COOH-terminal part of NUDE (residues 216–586) was deleted by excising the BglII-MfeI fragment (539 bp after filling in). This disrupts the ORF after residue 215 of NUDE and adds 16 new amino acid residues followed by a stop codon. Hybrids between the *nudE* gene and human and mouse cDNAs were made using recombination by PCR. The region of the *nudE* gene encoding residues 27–182 was replaced with the fragments of human cDNA clone (accession number AA424443) or *X. laevis* MP43 cDNA clone (accession number U95097). The replacing fragments encode 152-residue-long domains similar to the NUDE coiled-coil. The sequence of the used portion of the AA424443 clone was identical to the consensus derived from overlapping ESTs AA424443, R55738, AA442918, and H24090.

Protein Techniques

The affinity-purified polyclonal antibody against the NUDF protein was made by Xiang et al. (1995a). P5D4 mouse monoclonal antibody and protein G–coupled Sepharose 4B were from Sigma-Aldrich. 200 μl of protein G–Sepharose (packed volume) and 60 μl of P5D4 antibody solution (6.5 mg/ml of IgG) were coupled with dimethylpimelimidate as described (Harlow and Lane, 1988). Antigens were detected on immunoblots using an appropriate alkaline phosphatase conjugate and BCIP/NBT as a chromogenic substrate.

To make total protein extracts, *A. nidulans* cells from 18 h cultures were disrupted by grinding in liquid nitrogen and resuspended in 50 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 0.5% SDS. 1 ml of extraction buffer was added per gram of dry-pressed mycelium. The extracts were cleared by centrifugation for 10 min at 16,000 *g* and adjusted to the same protein concentration using the Bradford assay. Alternatively, the ground mycelium was resuspended in the SDS-PAGE loading buffer, boiled and cleared by centrifugation.

For immunoprecipitations, XX21 and XX21x6-17 strains were grown at 32°C for 18 h. 0.45 g of dry-pressed mycelium was disrupted by grinding in liquid nitrogen followed by addition of 0.45 ml of PBS containing protease inhibitor cocktail for fungal extracts (Sigma-Aldrich) and vortexing with glass beads. All subsequent steps were performed on ice or at 4°C. Extracts were cleared by centrifugation for 5 min at 10,000 *g* and incubated

for 2 h with 40 μl (packed volume) of protein G–Sepharose beads coupled to P5D4 monoclonal antibody in a total volume of 0.6 ml on a rocker. Beads were washed five times with 1 ml of PBS, boiled in 60 μl of SDS-PAGE loading buffer, and removed by centrifugation. 10-μl samples were analyzed by Western blotting and silver staining.

Other Techniques

A Gal4p-based yeast two-hybrid system Matchmaker 3, which includes *S. cerevisiae* strain AH109 and plasmids pGBKT7 and pGADT7, was purchased from CLONTECH Laboratories and used according to supplied instructions.

DAPI staining of nuclei and quantitation of nuclear migration was done essentially as described (Willins et al., 1995). The spores of the *nudF7* mutant were transformed with different multicopy plasmids, germinated under the most restrictive conditions (43°C, no KCl), and stained with DAPI when the majority of cells had 8–16 nuclei. Nuclear migration was scored for germlings with 8–16 nuclei. If a spore had several germtubes, only the one with the most nearly normal nuclear distribution was considered. Nuclear migration was scored as failed if fewer than three nuclei had entered the germtube. If three or more nuclei were observed in the germtube, but nuclei were not evenly distributed or an abnormally large cluster was present in the spore remnant, nuclear migration was scored as intermediate.

Results

Extra Copies of the nudE Gene Suppress the nudF7 and Other Conditional nud Mutants

We employed an *A. nidulans* autonomously replicating vector (Gems and Clutterbuck, 1993; Aleksenko and Clutterbuck, 1997) to screen for multicopy suppressors of the temperature sensitive *nudF7* mutation in *A. nidulans*. This vector maintains itself extrachromosomally in *A. nidulans* at ~10 copies per nucleus (Gems et al., 1991). The screen was based on partial complementation of the XX21 (*nudF7; pyrG89; yA2*) conidiation defect by genomic DNA fragments ligated to the *A. nidulans* replicating vector pAid. The XX21 strain is temperature sensitive due to the presence of the *nudF7* mutation. It grows like wild-type and produces yellow conidia (asexual spores) at the permissive temperature of 32°C, but forms smaller colonies and fails to produce conidia at the restrictive temperature of 43°C. Under semi-restrictive conditions, such as intermediate temperature (37°C) or at 43°C on high salt medium (0.6 M KCL), this strain grows almost to the same size as wild-type, but fails to conidiate and hence has the brownish color of unconidiating mycelium. We expected that a multicopy suppressor that improved the growth of the XX21 would also improve its conidiation. This would render the mycelium with the suppressor plasmid a yellow color that should be noticeable in the background of brownish, unconidiating mycelium.

Two genes were repeatedly identified in the screen. One was the *nudF6* allele of the *nudF* gene (the *nudF6* mutant was used as a source of DNA for the screen). Another gene was similar to *the N. crassa* nuclear migration gene *ro-11* (Minke et al., 1999). The *ro-11*-like gene was named *nudE*. Note, that *nud* mutations assigned to loci B, D, and E in the original screen (Morris, 1976) were subsequently mapped to the *nudA* gene (Morris, N.R., unpublished data). Multicopy plasmids with the *nudE* gene improve the growth, conidiation and nuclear distribution of the *nudF7* mutant (Fig. 1 A; Table I). Suppression of the *nudF6* mutation, which is tighter than *nudF7* (Xiang et al., 1995a),

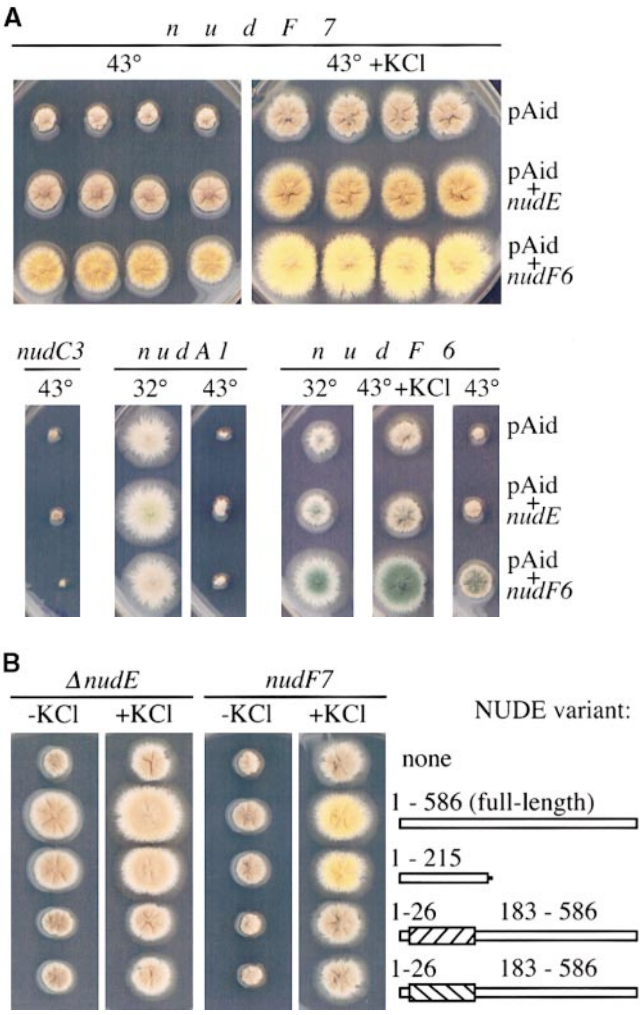

Figure 1. (A) Suppression of *nud* mutants by extra copies of the *nudE* and *nudF6* genes. The indicated mutants (all are conditional, temperature sensitive) were transformed with either the empty vector pAid or pAid clones bearing *nudE* and *nudF6* genes (plasmids recovered in the multicopy suppressor screen) and grown at 32°C. Transformants were gridded on YAG plates with or without KCl and incubated at 43°C or 32°C for 2 d. All strains have different color of conidia: yellow for *nudF7*, green (wild-type) for *nudF6*, chartreuse for *nudA1*, and white for *nudC3*. The intensity of the colony color is proportional to the number of conidia produced. Four independent transformants of the *nudF7* mutant are shown for each plasmid to demonstrate reproducibility of phenotypes. (B) Complementation of the *nudE* deletion and the *nudF7* mutant by extra copies of *nudE*, the *nudE* NH_2-terminal domain, and *nudE* chimeras carrying coiled-coil regions from human and frog proteins, respectively. Strains were transformed with *nudE* variants in pAid vector and grown at 43°C. Numbers refer to amino acid residues of NUDE protein expressed by the constructs (see Fig. 3 for detailed amino acid sequences). Due to the presence of a fawn color marker, conidiating and unconidiating *ΔnudE* colonies have very similar colors at this temperature.

was very weak. Extra copies of the *nudE* gene also suppressed the conidiation defect of the *nudA1* CDHC mutant grown under mildly restrictive conditions (32°C, no KCl, Fig. 1 A), but suppression of the *nudA1* mutant and of the *nudC3* mutant was barely detectable under the most

Table I. Effect of nudE Overexpression and Deletion on Nuclear Migration

Genotype	% nud	% intermed.	%wt
nudF7 [pAid]	52	43	5
nudF7 [pAid+*nudE*]	16	56	28
nudF7 [pAid+*nudF6*]	16	56	28
wild-type	0	0	100
ΔnudF	64	36	0
ΔnudE	21	67	12

A. nidulans conidia were germinated and stained with DAPI when the majority of germlings had 8–16 nuclei. The nuclear migration in germlings was scored as failed (nud), intermediate (intermed.), or wild-type (wt) as described in Materials and Methods. The nudF7 mutant carrying indicated plasmids was grown at 43°C. Wild-type and deletion strains were grown at 37°C.

restrictive conditions (43°C, no KCl). The conidiation defect of the *nudC3* mutant grown under mildly restrictive conditions was not suppressed, nor was the conidiation defect of another mutation that affects nuclear migration and conidiation, *apsA5* (Fischer and Timberlake, 1995; data not shown).

The amount of NUDF protein is reduced at the restrictive temperature in *nudF6* and *nudF7* mutants (Xiang et al., 1995a). However, suppression by *nudE* was not due to the affect on NUDF concentration. Extra copies of the *nudE* gene did not increase the level of NUDF protein in the *nudF7* mutant (Fig. 2). Predictably, extra copies of the *nudF6* gene increase the amount of the mutant NUDF protein (Fig. 2). Interestingly, we consistently observed inhibition of the *nudC3* mutant by extra copies of the *nudF6* allele (Fig. 1 A). This is opposite to the effect of the wild-type *nudF* gene, which is a multicopy suppressor of the *nudC3* mutation (Xiang et al., 1995a). A possible interpretation is that the overexpressed mutant NUDF protein sequesters mutant NUDC.

The *nudE* null mutant was viable, but displayed impaired nuclear migration, reduced colony growth, and a conidiation defect. All these defects were less severe than in the *ΔnudF* or *ΔnudA* mutants (Table I and Fig. 1 B), which have identical phenotypes (Willins et al., 1995). The double mutants *ΔnudE*;*ΔnudF* and *ΔnudE*;*ΔnudA* were similar to the *ΔnudF* and *ΔnudA* single mutants (data not shown), indicating that *nudE* affects the same pathway as *nudF* and CDHC. To make sure that differences between the *ΔnudE* and *ΔnudF* strains were not caused by background mutations, we crossed the *ΔnudE* strain to GR5, the wild-type parent of the *ΔnudF* strain. All progeny obtained from the cross were either wild-type or *ΔnudE*-like.

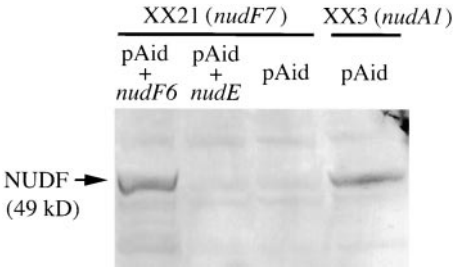

Figure 2. Extra copies of the *nudE* gene do not change the level of NUDF protein in the *nudF7* mutant. Total protein extracts were prepared from *nudF7* and *nudA1* mutants carrying indicated plasmids and NUDF protein levels were analyzed by Western blotting with the anti-NUDF antibody. Strains were grown at 43°C in YG medium. A *nudA1* mutant was included as a control of the wild-type level of NUDF. The amount of NUDF protein in the *nudF6* mutant is less than in *nudF7* (Xiang et al., 1995a).

Only the NH_2-terminal Coiled-Coil Domain of the NUDE Protein Is Absolutely Required for Its Function

The NUDE protein closely resembles the RO11 protein of *N. crassa* (Minke et al., 1999). The similarity is strongest within the first 190 residues, which are predicted to form a continuous coiled-coil structure (Fig. 3 A, see also Minke et al., 1999). The COOH-terminal region that follows the coiled-coil is poorly conserved between NUDE and RO11. In both proteins, however, it has an excess of positively charged residues (calculated pI = 12) and a high serine content (>18%). Other similar features of the COOH-terminal domain include a short central coiled-coil or an α-helix and the sequence DLGET(F,Y) at the very end.

A search of the public database for proteins similar to NUDE/RO11 turned up multiple sequences that had extensive homology with the coiled-coil in the NH_2-terminal part of the NUDE/RO11 but little or no homology with the rest of the molecule (Fig. 3 B). One was the mitotic phosphoprotein 43 (MP43) of *Xenopus laevis* (Stukenberg et al., 1997). Multiple human and mouse ESTs, apparently coming from at least two different genes, were also identified. The translation product of the human EST AA424443 that we chose to work with is almost identical (94% identity for the sequence shown in Fig. 3 B) to the rabbit endooligopeptidase A–related protein (Hayashi et al., 2000). A human cDNA encoding a similar coiled-coil (72% identity with AA424443) was recently annotated as HOM-TES-87 tumor antigen mRNA (accession number AF124431).

We constructed two chimeric nudE genes by substituting the sequence encoding the most conserved region of the NUDE coiled-coil (the region between arrowheads in Fig. 3 B) for the corresponding sequences from the human EST AA424443 and from the frog MP43 cDNA. Neither of the constructs was functional in *A. nidulans* (Fig. 1 B). A NUDE deletion variant with the in-frame deletion of residues 45–214 was also not functional (data not shown). The latter construct, when expressed as a fusion with green fluorescent protein, could be readily observed in live cells (Efimov, V.P., and N.R. Morris, manuscript in preparation), ruling out the possibility that the deletion variant was not expressed. Surprisingly, the NUDE construct containing the coiled-coil region alone complemented the nudE deletion and suppressed the nudF7 mutation (Fig. 1 B). Thus, the biological function of the NUDE protein is carried out by its NH_2-terminal coiled-coil, while the function of the COOH-terminal domain appears to be dispensable.

The NUDE Coiled-Coil Domain Interacts with the NUDF Protein and this Interaction Is Evolutionary Conserved

Multicopy suppressors often identify physically interacting proteins. We detected a NUDE-NUDF interaction in a Gal4p-based yeast two-hybrid system (Fig. 4). The same

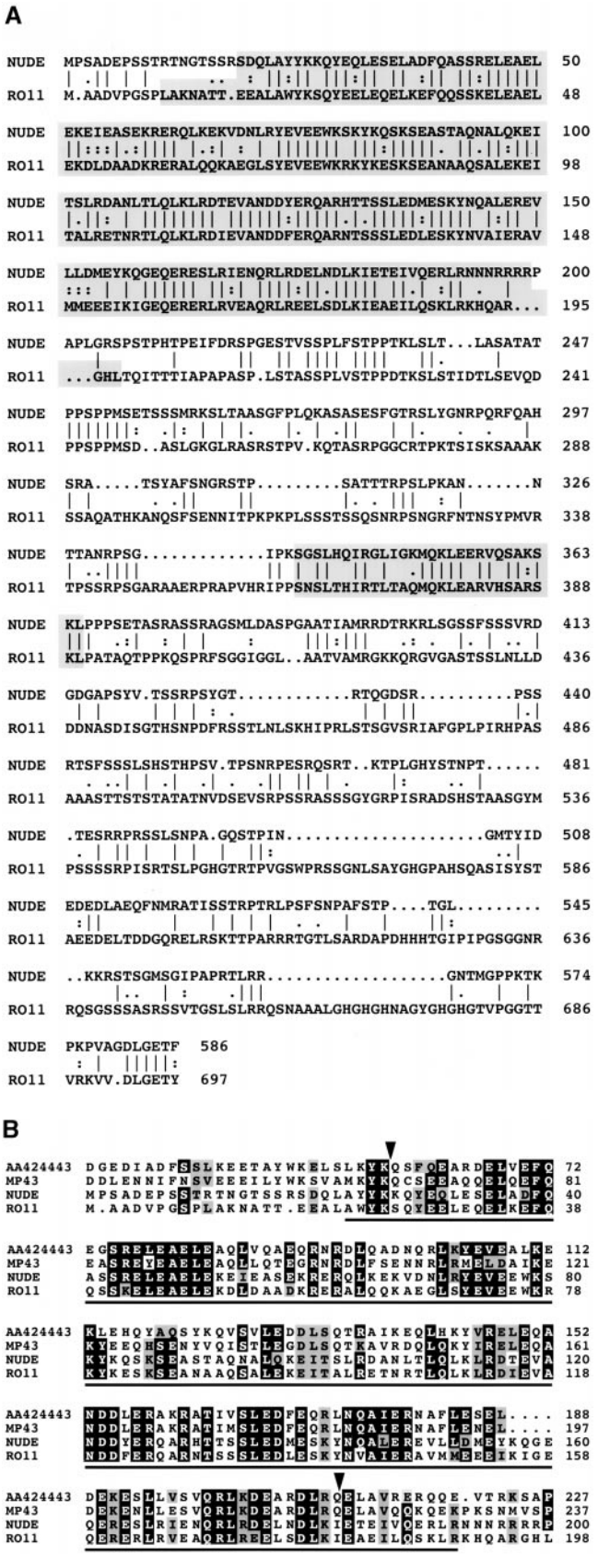

Figure 3. (A) Homology between *A. nidulans* NUDE protein and *N. crassa* RO11 protein. Shaded residues are predicted to form a coiled-coil structure. The probabilities of coiled-coil formation are 0.9–1 for the NH_2-terminal region and 0.7–0.8 for the central region. Probabilities of coiled-coil formation were calculated using program COILS version 2.1 (MTIDK matrix, unweighted a and d positions, window = 28) at http://www.ch.embnet.org/software/COILS_form.html (Lupas et al., 1991). Assigning 2.5-fold weights to a and d positions slightly changes the probabilities at coiled-coil ends and increases probability for the central coiled-coil to 0.9–1. (B) Alignment between coiled-coil regions of an ORF predicted from human EST with GenBank accession number AA424443, *X. laevis* MP43 (accession number U95097), *A. nidulans* NUDE (accession number AF085679), and *N. crassa* RO11 (accession number AF015560). The underlined residues are predicted to form coiled-coil structure with probabilities >0.9. The regions between arrowheads were used in sequence exchange experiments to test if human or *X. laevis* coiled-coil regions can substitute for the NUDE coiled-coil. The percentages of identical (similar) residues for the underlined coiled-coil regions are 69% (82%) between NUDE and RO11, 38% (51%) between NUDE and AA424443, 39% (51%) between NUDE and MP43, 72% (83%) between AA424443 and MP43, 40% (52%) between RO11 and MP43, 40% (52%) between RO11 and AA424443.

results were obtained with the full-length NUDE and its NH_2-terminal coiled-coil fragment (data not shown). The interaction appears to be weak as it can be detected only with the most sensitive reporter gene *HIS3*. The use of animal homologues of the NUDE and NUDF proteins provides a rigorous control for the specificity of this interaction. No interaction can be detected between *A. nidulans* NUDE and human LIS1 or between chimeras of NUDE carrying the coiled-coil regions from human or frog proteins and *A. nidulans* NUDF. On the other hand, the frog and human NUDE chimeras interact with human LIS1. The latter interactions are readily observed with both the *HIS3* and *ADE2* reporter genes. As expected, all coiled-coil constructs interact with themselves. Interestingly, the *Aspergillus* NUDE coiled-coil does not interact with the *Xenopus* or human coiled-coils, while the *Xenopus* and human coiled-coils interact with each other. These results demonstrate that the region delimited by amino acid residues 25–183 of the *A. nidulans* NUDE coiled-coil specifically binds NUDF protein, while the corresponding region of the human and frog NUDE/RO11 homologues binds human LIS1.

Finally, we tested whether NUDE and NUDF proteins interacted with each other physically in *A. nidulans* protein extracts. To facilitate detection of the NUDE protein, we tagged the *nudE* gene in the *A. nidulans* genome with six copies of the Vesicular Stomatitis Virus Glycoprotein (VSV-G) epitope sequence, which is recognized by the monoclonal antibody P5D4 (Soldati and Perriard, 1991). The tagged NUDE protein differs from the native protein by the extra sequence RS[YTDIEMNRLGK (GS)]$_6$TMG following glycine 569. Four independently obtained tagged strains were indistinguishable from the parent strain, indicating that the tagged gene was functional. The tagged NUDE::(VSV-G)$_6$ protein was readily detected in total protein extracts by Western blotting with the P5D4 antibody (Fig. 5 A). The protein is likely to be modified as it often migrates as a closely spaced doublet and can be further separated into at least four distinct bands (Fig. 5 B). Immunoprecipitations were performed with the P5D4 antibody using protein extracts from the tagged *nudE::(VSV-G)$_6$* strain and, as control, the parent *nudE*$^+$ strain. The NUDE::(VSV-G)$_6$ and

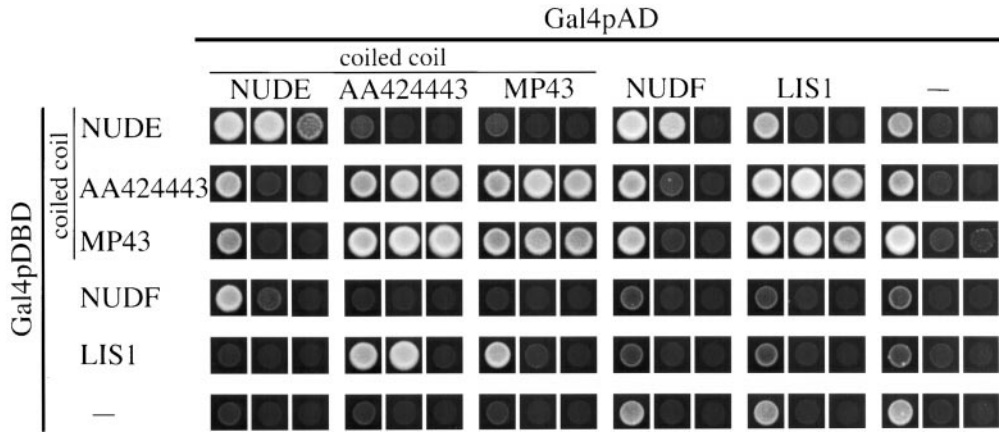

Figure 4. NUDE and NUDF proteins interact in a two-hybrid system. *S. cerevisiae* strain AH109 was transformed with pairwise combinations of plasmids expressing indicated proteins as fusions with either the Gal4p DNA binding (Gal4pDBD) or Gal4p activating (Gal4pAD) domains. NUDE, AA424443 and MP43 refer to the NH_2-terminal domains of *A. nidulans* NUDE protein (residues 1–195) and corresponding fusions with human and *X. laevis* homologues, respectively (Fig. 3 B). For each pair of plasmids, growth on three media is shown (from left to right): SD/-Leu/-Trp/-His; SD/-Leu/-Trp/-His with 3 mM 3-AT; SD/-Leu/-Trp/-Ade. The first two media select for the expression of the *HIS3* reporter gene, while the third medium selects for the expression of the *ADE2* reporter gene. Growth in the absence of histidine or adenine is expected to result from interactions between proteins encoded by plasmids. Note that Gal4pDBD fusions with the coiled-coil domains activate the *HIS3* gene expression in the absence of any interactions. 3 mM 3-AT (3-amino-1,2,4,-triazole), a competitive inhibitor of the *HIS3* gene product, suppresses the resulting background growth.

NUDF proteins were found to coprecipitate (Fig. 5 C), thus corroborating the results of the two-hybrid assay.

Discussion

We conducted a multicopy suppressor screen in the filamentous fungus *A. nidulans* to identify genes interacting with the *nudF* gene, a putative homologue of the human gene *LIS1*, which is implicated in brain development. The *nudE* gene, an apparent homologue of *N. crassa ro-11*, was obtained from the screen. Multicopy suppressors often correspond to physically interacting proteins. We observed a specific interaction between NUDE and NUDF in a yeast two-hybrid system (Fig. 4). In addition, an epitope-tagged NUDE coprecipitated with NUDF in *A. nidulans* protein extracts (Fig. 5 C). Thus, the simplest explanation for the suppression of conditional *nud* mutants by extra copies of the *nudE* gene is that increasing the level of the NUDE protein results in a more efficient formation of the NUDE-NUDF complex. NUDE-NUDF association is consistent with our observations that in live *A. nidulans* cells, full-length NUDE- and NUDF-GFP fusions localize to similar comet-like structures that move toward the hyphal tips (Xiang, X., V.P. Efimov, and N.R. Morris, manuscript in preparation). Similar structures are also observed with the CDHC-GFP fusion (Xiang et al., 2000). Given the size of the NUDE coiled-coil that binds NUDF (~170 residues or 25 nm in length) and its conservation between fungal, frog and human proteins (Fig. 3 B), it is likely to bind other proteins in addition to NUDF.

The closest match to the NUDE protein is the *N. crassa* nuclear migration protein RO11. Deletion of the *ro-11* gene in *N. crassa* causes the same nuclear distribution defect that is characteristic of dynein/dynactin mutants, without affecting cytoplasmic microtubules, dynein/dynactin accumulation at hyphal tips or the levels of CDHC (RO1) and $p150^{Glued}$ (RO3) (Minke et al., 1999). Deletion of the *A. nidulans nudE* gene similarly produced a viable strain with a nuclear distribution defect. The sequence similarity between NUDE and RO11 proteins and the involvement of both proteins in nuclear distribution indicate that NUDE and RO11 are functional homologues. Both proteins consist of two distinct domains: a highly conserved NH_2-terminal coiled-coil and a poorly conserved COOH-terminal tail. The importance of the NH_2-terminal coiled-coil domain is underscored by the fact that it is sufficient

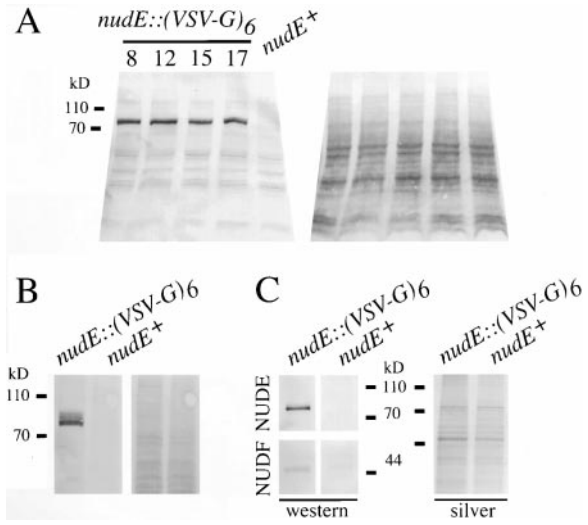

Figure 5. NUDE protein tagged with VSV-G epitopes coprecipitates with NUDF in *A. nidulans* protein extracts. (A) Total protein extracts from four independently isolated *NUDE::(VSV-G)$_6$* strains (#8, 12, 15, 17) and the parent strain with the wild-type *nudE* gene were analyzed by SDS-PAGE (4–20%) and immunoblotting with P5D4 antibody (left). Ponceau S staining of the membrane after protein transfer is shown on the right. (B) Same samples as in A (tagged strain #17 and untagged control) after better separation on a 10% SDS-PAGE. (C) The left panel shows immunoblotting of proteins precipitated by P5D4 antibodies coupled to Sepharose beads from *nudE::(VSV-G)$_6$* and *nudE$^+$* extracts with P5D4 and anti-NUDF antibodies. The right panel shows silver staining of the same samples.

for the biological activity of NUDE protein when expressed from a multicopy plasmid. However, it is possible that the extra copies of the gene make up for the lack of the COOH-terminal domain.

We selected the coiled-coil regions encoded by the *X. laevis* protein MP43 (Stukenberg et al., 1997) and by a human EST (accession number AA424443) as potential homologues of the NUDE/RO11 NH_2-terminal domain (Fig. 3 B). However, they were not functional in *A. nidulans* (Fig. 1 B). Also, we were not able to complement the *A. nidulans nudF* mutations with the human *LIS1* gene (Xiang, X., V.P. Efimov, and N.R. Morris, unpublished data), despite the 42% sequence identity between the two proteins. As an alternative, we used a yeast two-hybrid system to analyze interactions between *Aspergillus*, human, and *Xenopus* proteins (Fig. 4). The *A. nidulans* NUDE coiled-coil interacts with NUDF and itself, while NUDE chimeras carrying the coiled-coil regions from human or frog homologues interact with human LIS1 and themselves. The above interactions are extremely specific as *A. nidulans* NUDE does not interact with human LIS1 or human and frog NUDE homologues, nor does NUDF interact with the human or frog NUDE homologues.

While this work was in progress, we learned that a mouse NUDE/RO11-like protein was identified by Dr. S. Hirotsune's lab in a two-hybrid screen with the mouse LIS1 protein (Sasaki, S., A. Shionoya, M. Ishida, Y. Sugimoto, and S. Hirotsune, manuscript submitted for publication). At the amino acid level, the human EST fragment used by us (sequence in Fig. 3 B) has 99.5% identity with the mouse sequence. A slightly different sequence has been identified in the two-hybrid screen with the human LIS1 protein by Dr. C. Walsh's lab (Feng, Y., and C. Walsh, personal communication).

The region of the MP43 protein that follows the coiled-coil is much shorter than the equivalent region in the NUDE protein (160 residues vs. 394) and shows little sequence homology to NUDE. The salient features of this region in NUDE and RO11, an excess of positively charged residues and serines and the presence of a short central α-helix, are recognizably conserved in the MP43 COOH-terminal domain. It has a pI of 9.8, a serine content of 15%, and a predicted central α-helical region of ~50 residues. Considering all the facts, it is very likely that MP43 is a homologue of the NUDE/RO11 protein that functions in the cytoplasmic dynein pathway and interacts with the *X. laevis* homologue of LIS1 protein. As the MP43 protein is phosphorylated specifically during mitosis (Stukenberg et al., 1997), an exciting possibility is that its modification may be related to dynein/dynactin regulation. Cytoplasmic dynein drives the movement of membranous networks in *Xenopus* interphase extracts, and this movement ceases during mitosis, presumably because dynein dissociates from membranes (Niclas et al., 1996).

The sequence similarity between NUDF and LIS1, and the fact that both NUDF and LIS1 interact with the same domain of NUDE and its human homologue, respectively, support the idea that NUDF and LIS1 are functionally related. The documented involvement of fungal NUDF/Pac1p and NUDE/RO11 proteins in the cytoplasmic dynein pathway suggests that LIS1 exerts its effect through cytoplasmic dynein. Several hypothetical roles for cytoplasmic dynein in lissencephaly have been discussed (Sapir et al., 1997; Morris et al., 1998a,b; Vallee et al., 2000). They include a role in the regulation of microtubule dynamics, in mitosis, in nuclear translocation, and in retrograde transport of signaling molecules. Of particular interest is the recent finding that in axons cytoplasmic dynein generates a force counterbalancing contractile forces generated by myosin (Ahmad et al., 2000).

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