The L279P Mutation of Nuclear Distribution Gene C (NudC) Influences Its Chaperone Activity and Lissencephaly Protein 1 (LIS1) Stability*

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LIS1, a gene mutated in classical lissencephaly, plays essential roles in cytoplasmic dynein regulation, mitosis and cell migration. However, the regulation of LIS1 (lissencephaly protein 1) protein remains largely unknown. Genetic studies in Aspergillus nidulans have uncovered that the Nud (nuclear distribution) gene is able to suppress the phenotypes of nudF, an Aspergillus ortholog of LIS1. Here, we showed that L146 in Aspergillus NudC and its flanking region were highly conservative during evolution. The similar mutation in human NudC (L279P) obviously led to reduced LIS1 and cellular phenotypes similar to those of LIS1 down-regulation. To explore the underlying mechanism, we found that the p23 domain-containing protein NudC bound to the molecular chaperone Hsp90, which is also associated with LIS1. Inhibition of Hsp90 chaperone function by either geldanamycin or radicicol resulted in a decrease in LIS1 levels. Ectopic expression of Hsp90 partially reversed the degradation of LIS1 caused by overexpression of NudC-L279P. Furthermore, NudC was found to regulate the ATPase activity of Hsp90, which was repressed by the mutation of L279P. Interestingly, NudC itself was shown to possess a chaperone function, which also was suppressed by the L279P mutation. Together, these data suggest that NudC may be involved in the regulation of LIS1 stability by its chaperone function.

Mutations in a single allele of the human LIS1 gene cause type I lissencephaly, a severe brain malformation characterized by smooth cerebral surface and disorganized cortical layering (1, 2). LIS1, the product of the LIS1 gene, has been reported to be essential for cytoplasmic dynein regulation, mitosis, and cell migration (3–5). LIS1 not only dynamically co-localizes with cytoplasmic dynein during various biological processes but also directly binds to multiple sites within dynein heavy chain (6–10). Purified recombinant LIS1 is able to increase the microtubule-stimulated ATPase activity of the dynein motor in vitro and suppress the motility of dynein on microtubules (11, 12). Either up-regulation or down-regulation of LIS1 interferes with mitotic progression of neuronal and non-neuronal cells and consequently inhibits cell proliferation and survival in a range of organisms from yeast to mammals (6, 13–19). A number of data from both LIS1-deficient mice and cells treated with RNAi strongly suggest a key role of LIS1 in neuronal migration during development of the cerebral cortex (14, 15, 17, 19–22). However, the regulation of LIS1 protein is still largely unclear.

Genetic studies in the filamentous fungus Aspergillus nidulans have uncovered an evolutionarily conserved pathway, Nud (nuclear distribution) pathway, which is involved in the regulation of the cytoplasmic dynein complex (23). Observation on this fungus shows that the nuclei move apart after mitosis, and then migrate toward the hyphal tip, resulting in a relatively even distribution along the mycelium (23). A temperature-sensitive mutation in the nudC gene (nudC3) of A. nidulans specifically causes a strikingly uneven distribution of nuclei along the mycelium at the restrictive temperature (24). Meanwhile, this mutation substantially reduces the protein level of NudF, an Aspergillus ortholog of LIS1, and ectopic expression of the nudF gene is able to suppress the phenotypes of nudC3 mutation (13). All of the suppressors of the nudC3 mutation reverse its temperature-sensitive phenotype by restoring the intracellular concentration of the NudF protein (25). These results suggest that NudC (nuclear distribution gene C) may be an upstream regulator of NudF in A. nidulans.

In 1997, Chiu and Morris (26) demonstrated that the nudC3 has a missense mutation at amino acid 146 that causes leucine to be replaced by proline, which can be corrected by sncB69 (suppressor of nudC B69), a mutant tRNA(Leu). The sncB69 mutation loses a single nucleotide in the anticodon of a tRNA(Leu) that changes its normal leucine anticodon to the proline anticodon. This mutation may permit incorporation of leucine at the mutant nudC3 proline codon 146 and thereby suppress the nudC3 mutant phenotype. These data indicate that the L146P mutation of NudC leads to reduced LIS1 and consequently results in nuclear migration defects in Aspergillus.

Here, we determine whether a similar event occurs in mammals. Our data showed that Leu146 in Aspergillus NudC is con-
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served to Leu279 in human NudC. Overexpression of NudC-L279P in mammalian cells led to a decrease of LIS1 level. The p23 domain-containing protein NudC not only forms a biochemical complex with LIS1 and the molecular chaperone Hsp90 (heat shock protein 90) but also influences the ATPase activity of Hsp90, suggesting that NudC may be a potential Hsp90 co-chaperone. Furthermore, inhibition of Hsp90 chaperone function results in a decrease in LIS1 levels, and ectopic expression of Hsp90 reverses the degradation of LIS1 caused by the L279P mutation of NudC, implying LIS1 as a possible client protein for Hsp90. These data, for the first time, indicate that NudC may be a potential Hsp90 co-chaperone to stabilize LIS1.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Human NudC cDNA was amplified by RT-PCR and subcloned into pGEX5X-1 (GST fusion vector, Amersham Biosciences Pharmacia), pET-28a (His-tag vector, Novagen, Madison, WI), pCMV-Tag2C (FLAG epitope vector, Stratagene, La Jolla, CA) and pGFP-C1 (GFP fusion vector, Clontech, Palo Alto, CA), respectively. GST-NudC-C3, FLAG-NudC-L279P, FLAG-NudC-C and FLAG-NudC-N also were separately constructed. Human HSP90α cloned by RT-PCR were subcloned into pET-28a and pCMV-Tag2C vectors. FLAG-NudC-L279P, FLAG-NudCΔP23, GFP-NudC-L279P, and His-NudC-L279P were constructed by PCR-based site-directed mutagenesis. p23 cDNA also was cloned into pET-28a vector. The sequences of all constructs were confirmed by DNA sequencing.

Cell Culture and DNA Transfection—HeLa cells were maintained in DMEM containing 10% fetal bovine serum (Invitrogen). Cells were transfected with the indicated constructs by
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**FIGURE 2.** The mutation of L279P in NudC inhibits its interaction with LIS1. A, NudC associates with LIS1 in vitro. GST pulldown assay was performed by incubating purified GST-fused proteins with lysates of HeLa cells. LIS1, which interacts with either NudC or its fragments, was detected by immunoblotting analysis. The input proteins were stained with Coomassie Brilliant Blue. B, NudC co-immunoprecipitates with LIS1 in vivo. HeLa cells transfected with the indicated vectors were subjected to immunoprecipitation analysis with anti-FLAG antibody-coupled beads. Western analyses were performed with anti-FLAG and LIS1 antibodies. C, the L279P mutation of NudC suppresses the interaction between NudC and LIS1 in vitro. Purified GST-fused proteins were incubated with the lysates of HeLa cells. LIS1, which binds to either GST-NudC or GST-NudC-L279P, was determined by immunoblotting with anti-LIS1 antibody. The input proteins were visualized with Coomassie Brilliant Blue. D, the mutation of L279P in NudC inhibits the association of NudC with LIS1 in vivo. HeLa cells transfected with the indicated vectors were subjected to co-immunoprecipitation analysis with anti-FLAG antibody-coupled beads. Western analyses were performed with the antibodies as shown.

GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA).

**Protein Expression and Purification**—Recombinant GST-fused proteins generated in *Escherichia coli* BL21(DE3) were purified by incubation with glutathione-agarose 4B beads (GE Healthcare) followed by elution with glutathione elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0). Recombinant His-tagged proteins produced in *E. coli* BL21 were purified by incubation with nickel-nitrilotriacetic acid beads (Qiagen) as described previously (27). The concentrations of proteins were determined by the BCA protein assay kit (Pierce).

**GST Pulldown Assays, Immunoprecipitation, and Western Blotting Analyses**—GST pulldown and immunoprecipitation assays were performed as described previously (28, 29). The immunoprecipitates or total proteins isolated from mammalian cells were resolved by SDS-PAGE and subjected to immunoblotting analyses with anti-NudC (28), Hsp90α, ERK2 (extracellular signal-regulated kinase 2), GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Sigma), FLAG (M2), LIS1 (Sigma), and GFP (Zymed Laboratories, Inc., San Francisco, CA) antibodies, and the according horseradish peroxidase-linked secondary antibodies (Amersham Biosciences Pharmacia).

**Immunofluorescence Staining**—HeLa cells grown on coverslips were fixed with cold methanol (−20 °C) and then stained with either anti-γ-tubulin or α-tubulin antibodies (Sigma), followed by incubation with Cy3-conjugated anti-mouse Ig secondary antibody (Jackson ImmunoResearch Laboratory).

Proteins identified in both the pCMV-Tag 2C and FLAG-NudC groups were excluded from the results. The obtained NudC-interacting proteins were classified according to Gene Ontology categories (UniProtKB).

**Drug Treatments**—Geldanamycin (InvivoGen, San Diego, CA) and radicicol (Sigma) were stored in the dark at −20 °C as a stock solution of 1.78 mM in dimethyl sulfoxide and 2.74 mM in ethanol, respectively. Immediately prior to use, either geldanamycin or radicicol was prepared in culture medium. Cells were incubated with either Hsp90 inhibitors or the corresponding control reagents in different concentrations for various periods as described in the text.

**Hsp90 ATPase Assay**—To completely remove the inorganic phosphate, all of the proteins for Hsp90 ATPase were fully dialedyzed into the buffer (50 mM Tris, 1 mM dithiothreitol, pH 7.4). ATPase assays were performed in reaction buffer (50 mM Tris, pH 7.4, 20 mM KCl, 6 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM ATP) and 1 μM Hsp90 at 37 °C with 2 μM NudC, NudC-L279P, or p23 for 20 min. The released inorganic phosphate was determined by measuring the absorbance at 650 nm using Cyto Phosphate Assay BIOCHEM kit (Cytoskeleton, Inc., Denver, CO). The remaining ATPase activity in the presence of 60 μM geldanamycin was interpreted as background and thus was subtracted from all of the total activities (27, 32).

**Citrate Synthase Aggregation Assay**—The aggregation reaction of citrate synthase (Sigma) was performed as described previously (33). Briefly, citrate synthase (0.15 μM) was incubated at 43 °C for 30 min either alone or in the presence of BSA.
(0.75 μM), NudC, NudC-L279P, or Hsp90 (0.15 μM each). To monitor kinetics of thermal aggregation, light scattering was measured in a DU 800 spectrophotometer (Beckman, Fullerton, CA) at 370 nm.

RESULTS

The L279P Mutation of NudC Leads to LIS1 Instability—In *A. nidulans*, the mutation of L146P in NudC (nudC3) greatly decreases the concentration of NudF protein at non-permissive temperature (26). Here, we investigated whether a similar event happened in mammals. Sequence analysis showed that both Leu146 and its flanking amino acid residues in *Aspergillus* NudC were conserved to Leu279 and its neighboring residues in human NudC (Fig. 1A). Western blotting revealed that overexpression of human NudC-L279P in HeLa cells caused a significant decrease in LIS1 protein levels, whereas the level of ERK2 remained unchanged (Fig. 1B), suggesting a conservative function of NudC during evolution. Furthermore, overexpression of NudC-L279P also led to cellular phenotypes similar to LIS1 deficiency, such as perinuclear accumulation of microtubules and enlarged distance between the centrosome and nucleus (Fig. 1, C–F) (9, 15).

The L279P Mutation of NudC Suppresses Its Binding to LIS1—Previous studies have demonstrated that mammalian NudC forms a biochemical complex with LIS1 in extracts from both mouse brain and HeLa cells (34). Here, our data confirmed the association of NudC with LIS1 in *in vitro* and *in vivo* (Fig. 2, A and B). Both of N and C termini of NudC contributed to the interaction between NudC and LIS1 (Fig. 2, A and B). Overexpression of NudC-L279P effectively inhibited the association of NudC with LIS1 in *in vitro* and *in vivo* (Fig. 2, C and D), suggesting that Leu279 of human NudC is important for its interaction with LIS1, which may contribute to the instability of LIS1.

Identification of NudC-binding Proteins—To elucidate the underlying mechanism for the regulation of LIS1 by NudC, we investigated the NudC-interacting proteins in HeLa cells. We performed mass spectrometry analysis of proteins co-purified with NudC immunoprecipitation. The data indicated that NudC appeared to be associated with 130 different proteins involved in various cellular processes, such as signal transduction, trafficking, chaperones, cytoskeleton, protein biosynthesis, nucleic acid binding, metabolic enzymes, and proteolysis (supplemental Table S1 and Fig. S1), implying NudC may have diverse functions in mammals.

The Interaction among NudC, LIS1, and Hsp90—Because NudC contains a conserved p23 domain, the key domain for Hsp90 co-chaperone p23 to regulate its chaperone function (27, 35, 36), and our data indicated that Hsp90 might be a NudC-interacting protein (supplemental Table S1); therefore,
we determined to examine whether NudC was associated with Hsp90. GST pulldown and co-immunoprecipitation assays showed that NudC indeed bound to Hsp90 (Fig. 3, A and B). Cell lysates were subjected to immunoblotting analyses with the antibodies as shown. Actin was used as a loading control. The intensities of LIS1 and actin bands were quantified using NIH ImageJ software. Relative abundances of LIS1 to actin were shown. Data are presented as the mean ± S.D. of three independent experiments. Asterisks indicate p < 0.05 compared with the corresponding controls (Student’s t test). DMSO, dimethyl sulfoxide.

FIGURE 4. Inhibition of Hsp90 chaperone function leads to reduction of LIS1. HeLa cells were treated with different concentrations of either geldanamycin or radicil for 48 h (A and C) or 3.56 µM geldanamycin or radicil for different times (B and D). Cell lysates were subjected to immunoblotting analyses with the antibodies as shown. Actin was used as a loading control. The intensities of LIS1 and actin bands were quantified using NIH ImageJ software. Relative abundances of LIS1 to actin were shown. Data are presented as the mean ± S.D. of three independent experiments. Asterisks indicate p < 0.05 compared with the corresponding controls (Student’s t test). DMSO, dimethyl sulfoxide.

FIGURE 5. Ectopic expression of Hsp90 partially counteracts the down-regulation of LIS1 caused by NudC-L279P overexpression. HeLa cells transfected with the indicated vectors for 60 h were subjected to immunoblotting analysis with the antibodies as shown. GAPDH was used as a loading control. Band intensities were quantified using ImageJ software (NIH). Relative abundances of LIS1 to GAPDH were shown. Data are presented as the mean ± S.D. of three independent experiments. Statistical significance was determined by Student’s t test (*, p < 0.05).

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FLAG-NudCΔp23 that lacks the p23 domain and transfected it into HeLa cells. Immunoprecipitation data showed that NudCΔp23 failed to bind to Hsp90 (supplemental Fig. S2), suggesting that the p23 domain may be required for the interaction between NudC and Hsp90. Nonetheless, the L279P mutation of NudC failed to affect its association with Hsp90 (Fig. 3, A and B).

As NudC interacts with both Hsp90 and LIS1, we investigated the direct association of Hsp90 with LIS1. GST pulldown assay and co-immunoprecipitation analysis showed that LIS1 bound to Hsp90 (Fig. 3, C and D). Further data revealed that endogenous LIS1 was able to bind to endogenous NudC and Hsp90 in vivo (Fig. 3E), suggesting that LIS1 forms a biochemical complex with Hsp90 and NudC. However, the L279P mutation of NudC appeared to have no significant effect on the interaction between Hsp90 and LIS1 (Fig. 3F).

Hsp90 Is Involved in Regulation of LIS1 Stability—Because LIS1 interacts with both Hsp90 and NudC, we determined whether Hsp90 function influences the regulation of LIS1. We employed two Hsp90 inhibitors, geldanamycin and radicil, which block the Hsp90 ATP-driven chaperone cycle and lead to proteasomal degradation of Hsp90 client proteins (37, 38). The results showed that inhibition of Hsp90 chaperone function by either geldanamycin or radicil induced LIS1 instability in a dose- and time-dependent manner (Fig. 4, A–D), suggesting that Hsp90 may be involved in LIS1 stability.

Because both Hsp90 inhibition and the L279P mutation of NudC led to LIS1 instability, we attempted to discover whether Hsp90 influences LIS1 reduction induced by the NudC L279P mutation. The data showed that ectopic expression of Hsp90 partially reversed the protein levels of LIS1 in cells overexpressing NudC-L279P (p < 0.05) (Fig. 5), indicating that Hsp90 may play a role in the regulation of LIS1 by NudC.

L279P Mutation of NudC Impairs Its Chaperoning Activity—The chaperone activity of Hsp90 has been reported to be usually regulated by its co-chaperones (27, 38, 39). The co-chaperone p23 plays a critical role in the Hsp90 chaperone cycle to stabilize the client protein-Hsp90 complex (40). Studies have shown that p23 is capable of inhibiting the ATPase activity of Hsp90 in vitro (35). Because NudC not only interacts with Hsp90 but also contains a p23 domain, we sought to examine whether NudC influences Hsp90 ATPase activity. Recombinant His-tagged NudC, NudC-L279P, p23, and Hsp90 proteins generated from bacteria were affinity-purified, respectively (Fig. 6A). The in vitro Hsp90 ATPase assays demonstrated that NudC effectively inhibited the ATPase activity of Hsp90.
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FIGURE 6. NudC and its L249P mutation influence the in vitro ATPase activity of Hsp90. A, His-tagged p23, Hsp90, NudC-L279P, and NudC proteins were purified by nickel-nitritoltriacetic acid beads and subjected to SDS-PAGE with Coomassie Blue staining. B, the in vitro ATPase activities of Hsp90 were examined in the absence or presence of geldanamycin (GA), NudC, NudC-L279P, or p23. All ATPase activities were expressed as the relative to that with Hsp90 only. The remaining ATPase activities in the presence of geldanamycin were interpreted to be background and were subtracted from all of ATPase activities. Data are representative of at least three independent experiments and are expressed as the mean ± S.D. Significance was determined by one-way analysis of variance. **, p < 0.01 and *, p < 0.05.

FIGURE 7. The mutation of L279P inhibits the ATP-independent chaperone activity of NudC. The chaperone activities were determined by the heat-induced citrate synthase (CS) aggregation experiment. Citrate synthase was incubated at 43 °C for 30 min either alone or in the presence of BSA, NudC, NudC-L279P, or Hsp90. Aggregation was determined by light scattering at 370 nm. Data were calculated as a percentage of the maximum aggregation of citrate synthase after 30 min for each trial. Data are expressed as the mean ± S.D. and representative of at least three independent experiments.

(p < 0.01, Fig. 6B). Interestingly, the inhibitory effect of NudC-L279P on Hsp90 ATPase activity is significantly weaker than that of wild-type NudC (p < 0.05, Fig. 6B). Furthermore, NudC and NudC-L279P exhibited the similar effects on the in vitro ATPase activities of eukaryotic Hsp90 purified from insect cells (supplemental Fig. S3).

Many of the Hsp90 co-chaperones have been found to have their own in vitro ATP-independent chaperone activity (41, 42). Recently, NUD-1, the Caenorhabditis elegans homolog of NudC, has been reported to possess in vitro chaperone activity (33). Therefore, we examined whether NudC itself also has an ATP-independent chaperone activity. The data revealed that NudC inhibits the heat-induced aggregation of citrate synthase nearly as efficiently as the Hsp90 chaperone, which was significantly inhibited by the mutation of L279P in NudC (Fig. 7), suggesting that Leu279 is indispensable for the chaperone activity of NudC.

DISCUSSION

In the present study, we provide evidence that mutation of L279P in mammalian NudC leads to instability of LIS1 that forms a complex with NudC and Hsp90. Inhibition of Hsp90 chaperone function reduces LIS1 level. Ectopic expression of Hsp90 partially counteracts LIS1 reduction caused by NudC-L279P mutation. Furthermore, NudC effectively inhibits Hsp90 ATPase activity in vitro, which is suppressed by the mutation of L279P. NudC itself has an ATP-independent chaperone function, which was also repressed by the L279P mutation. Thus, these results indicate that NudC may be involved in the regulation of LIS1 by Hsp90.

*NudC as a Potential Candidate for Hsp90 Co-chaperone To Influence LIS1 Stability—*The molecular chaperone Hsp90 is required for conformational regulation of a variety of client proteins, many of which are involved in cell signaling, such as protein kinases and transcription factors, including steroid hormone receptors (40, 43–45). Hsp90-dependent folding and activation of client proteins needs a range of accessory factors, or co-chaperones that assist Hsp90 either in client recognition or in the transition of client to its final active state (32, 35, 38, 39, 46, 47). In general, Hsp90 co-chaperones are found frequently—in vitro ATPase activity (44, 48). Here, our data clearly showed that NudC not only interacted with Hsp90 but also influenced the in vitro ATPase activity of Hsp90, which is consistent with the role of the co-chaperone p23 in the regulation of Hsp90 (35). Thus, these results suggest that NudC may function as a potential Hsp90 co-chaperone.

In A. nidulans, the nudC3 mutation greatly decreases the concentration of NudF protein at nonpermissive temperature, which can be rescued by extra copies of nudF gene, suggesting that NudC may be involved in the regulation of NudF (25). In mammals, overexpression of the NudC-L279P mutant that mimics nudC3 mutation led to significant degradation of LIS1, which was reversed by ectopic expression of Hsp90. Inhibition of Hsp90 function by either geldanamycin or radicicol also induced the instability of LIS1, an Hsp90-interacting protein, implying LIS1 may be an Hsp90 client protein. Moreover, the L279P mutation of NudC impaired its binding to LIS1 and its inhibition of Hsp90 ATPase activity. Taken together with the fact that LIS1, Hsp90, and NudC were able to form a biochemical complex, these data indicate that LIS1 may be regulated by NudC via Hsp90 chaperone. Further studies clearly will be needed to explore the underlying molecular details in this complex.

ATP-independent Chaperone Activity of NudC—Some proteins containing p23 domains have been shown to exhibit ATP-
independent in vitro chaperone activity (49). For example, p23 can bind to partially folded proteins, prevent their aggregation, and assist in their folding (49–51). The p23 domain-containing protein Sgt1 suppresses the thermal aggregation process of citrate synthase as efficiently as p23 protein (52). NUD-1, a C. elegans ortholog of mammalian NudC, also possesses such chaperone activity (33). Interestingly, a plant NudC homolog Bobber1 that regulates Arabidopsis development and thermotolerance as a potential small heat shock protein appears to prevent thermal aggregation of malate dehydrogenase in vitro (53, 54). Here, our results revealed that NudC significantly inhibited the heat-induced aggregation of citrate synthase, indicating that NudC may have an ATP-independent protein chaperone activity, similar to p23 and other p23 domain-containing proteins (54, 55).

The Multiple Functions of NudC—NudC has been reported to play a role in mitosis and cytokinesis in various organisms from fungus to human (56). Deletion of Aspergillus NudC affects the composition and morphology of the cell wall and is lethal (57). In C. elegans, silencing of nud-1 leads to a loss of midzone microtubules and the rapid regression of the cleavage furrow (58). We and others (28, 58) have shown that down-regulation of mammalian NudC results in multiple defects in mitosis and cytokinesis. Recently, mammalian NudC has been found to play an essential role in the anterograde transport of cytoplasmic dynein and dynactin by kinesin-1 (59). These data suggest that NudC may have multiple functions. Our mass spectrometry-based analysis of NudC-interacting proteins indicates that NudC associates with a number of proteins that participate in various biological processes, such as signal transduction, protein folding, nucleic acid binding, protein biosynthesis, metabolism, and other cellular processes. Some of them, such as mitogen-activated protein kinase kinase kinase 3 and protein arginine methyltransferase 5, have been found to be Hsp90-interacting proteins (60–61). Thus, it would be very interesting to determine whether NudC is involved in the regulation of these proteins.

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