Mutations in one allele of the human LIS1 gene cause a severe brain malformation, lissencephaly. Although most LIS1 mutations involve deletions, several point mutations with a single amino acid alteration were described. Patients carrying these mutations reveal variable phenotypic manifestations. We have analyzed the functional importance of these point mutations by examining protein stability, folding, intracellular localization, and protein-protein interactions. Our data suggest that the mutated proteins were affected at different levels, and no single assay could be used to predict the lissencephaly phenotype. Most interesting are those mutants that retain partial folding and interaction with specific interacting proteins. Overexpression of the PAF-AH α1 subunit dissolved aggregates induced by this mutant protein and increased its half-life. Overexpression of NudE or NudEL localized this mutant protein to spindle poles and kinetochores but had no effect on protein stability. Our results implicate that there are probably different biochemical and cellular mechanisms obstructed in each patient yielding the varied lissencephaly phenotypes.

The functional complexity of the vertebrate cerebral cortex is facilitated by a complicated structural organization. The developmental details of these features are therefore of critical interest. A singular feature of cortical development is the generation of neurons at sites distant from their place of terminal differentiation. Abnormalities in the migration of neurons into the embryonic cortex lead to loss of normal convolutions of the human neocortex, known as lissencephaly. The migration of neurons from the region of the cerebellar vermis, an unusual finding for patients with pachygyria with the additional feature of moderate hypoplasia. LIS1 contains an N-terminal region of 94 amino acids that includes a Lis-H domain. The patient with grade 4a (1) lissencephaly manifested by generalized increasing severity. Grade 5 is mixed pachygyria and SBH, whereas grade 6 is SBH alone. Deletions involving LIS1 are far more common than other mutations (8, 9); 88% result in a truncated or internally deleted protein. Up to now, only five missense mutations have been found in patients (9, 10). Our previous analysis indicated that truncated or internally deleted LIS1 protein is unlikely to fold correctly (11). This finding was supported by a study done in cells derived from lissencephaly patients, where no protein was detected from mutated alleles that were expected to result in a truncated protein (12). The five patients with point mutations that lead to a single amino acid substitution in LIS1 exhibit a variable phenotypic manifestation. Thus, the patients are classified from grade 3a to grade 6 (13–15). LIS1 is a member of the WD (tryptophan-aspartic acid) repeat family of proteins (1), as deduced from its amino acid sequence. A hallmark of this family is their involvement in multiple protein-protein interactions (16), and LIS1 is not an exception (reviewed in Ref. 17). LIS1 contains an N-terminal region of 94 amino acids that includes a Lis-H domain (LIS1 homology domain amino acids 8–37) (18), a coiled-coil domain (amino acids 51–79) important for dimerization (19), and seven WD repeats. Interestingly, the five reported mutations occur in conserved domains (15). The mutation F31S (10) is located in the Lis-H domain. The patient has grade 4a(1) lissencephaly manifested by generalized pachygyria with the additional feature of moderate hypoplasia of the cerebellar vermis, an unusual finding for patients with LIS1 mutations. Three mutations, H149R (13), G162S (10), and in the X-linked doublecortin (DCX) (2–4). Lissencephaly (smooth brain) is a severe abnormality of neuronal migration characterized by absent (agyria) or decreased (pachygyria) convolutions, producing a smooth cerebral surface with thickened cortex (5). Subcortical band heterotopia (SBH) is a related disorder in which there are bilateral bands of gray matter interposed in the white matter between the cortex and the lateral ventricles (6). SBH is very common among females with mutations in DCX (2, 3). Lissencephaly and SBH have been observed in different regions of the same brain, indicating an “agyria-pachygyria-band” spectrum. A grading system characterizes the malformations from grade 1 (most severe) to grade 6 (least severe) (7). Grades 1–4 are all lissencephalies of decreasing severity. Grade 5 is mixed pachygyria and SBH, whereas grade 6 is SBH alone.
S169P (14), cluster in WD2 with variable phenotypes. The patient with mutation H149R has the most severe LIS grade and clinical phenotype of the five patients with missense mutations (grade 3a). The patient with mutation G162S has an unusually mild form of LIS (grade 4a(2)), with a normal IQ, infrequent seizures, and mild clumsiness as his only motor deficit. The patient with mutation S169P is the only known patient with subcortical band heterotopia secondary to a LIS1 mutation and is thus classified as grade 6a. It has been suggested that the patient exhibits somatic mosaicism (14). The fifth mutation, D317H (14), is located in WD5 (grade 4a(1)) manifested by generalized pachygyria.

LIS1-protein interactions may be grouped conceptually into two classes: evolutionarily conserved and relatively new interactions. Much can be learned from the interactions that are conserved throughout evolution (20). NudF, a LIS1 homolog in Aspergillus nidulans, was identified in a screen for mutants defective in nuclear migration (21). NudC, which controls NudF levels (21), and nudE, a multicopy suppressor of mutated nudF (22), both have mammalian homologs that interact with LIS1 (22–27). Other gene products involved in the same pathway are subunits of the microtubule-based motor protein cytoplasmic dynein, or its regulatory complex dynactin (28–30). LIS1 interacts with subunits of the dynein and dynactin complexes in mammalian cells (25, 31–34). LIS1 also interacts with tubulin (34) and can modulate microtubule dynamics in vitro (35). Furthermore, LIS1 colocalize with NudC, NudEL, mNudE, and γ-tubulin at the centrosome and could control the microtubule nucleation (25, 26, 36). All these results indicate a conserved mechanism from nuclear movement in the fungus and flies to neuronal migration in the brain. An additional LIS1-interacting protein that is functioning in the dynein/dynactin pathway is CLIP-170 (37). We also detected an interaction between LIS1 and doublecortin (DCX) that mutations in the gene product also result in lissencephaly (38).

Another well-documented interaction is between LIS1 and the two catalytic subunits (α1 and α2) of platelet-activating factor acetylhydrolase (PAF-AH) Ib (39, 40). LIS1 is believed to act as the regulatory unit of this enzymatic complex. Based on the Drosophila homologs, this interaction is relatively new in evolution, as Drosophila α-subunit lacks both catalytic activity and the ability to interact with Drosophila LIS1 (41).

Taking into consideration our current knowledge regarding LIS1 function, we believe it is important to analyze how these five missense mutations affect protein half-life, cellular localization, and protein–protein interaction. That will promote our understanding as to how the disease is manifested in lissencephaly patients.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Culturing and transfection of HeLa and HEK293 cells were performed as described previously (37).

**Immunological Methods**—Cells were fixed in 100% methanol plus 1 mM EGTA for 10 min at –20 °C, followed by 15 min in 4% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM magnesium acetate, pH 6.9). They were washed three times for 5 min each in PBS (phosphate-buffered saline) and further permeabilized for 25 min in 0.1% Triton X-100 in PBS. The cells were treated with 50 mM NH4Cl in PBS for 10 min, washed three times for 5 min each in PBS, blocked in PBS-BSA (PBS containing 0.1% BSA), and labeled with the polyclonal anti-CLIP-170 antibody, TA (37), a monoclonal anti-EB1 antibody (Transduction Laboratories, E46420/Lot 1), or c-Myc antibody 9E10 clone from Sigma, antibodies to placcamycin containing antifading agent 1,4-diazabicyclo(2.2.2)octane (Sigma) at 100 mg ml⁻¹. Pictures of fixed cells were collected using a three-dimensional deconvolution imaging system, or using Olympus IX-70 microscope with a digital camera.

**Constructs**—Site-directed mutagenesis was performed using PCR in LIS1-FLAG-DeRed or pAGA-LIS1 vectors. Based on the patients’ mutations (13–15), we synthesized the following oligonucleotides: F31S, 5′-gaggattatcatgcttttacaagagaaggtaatgct; H149F, 5′-caattaagaggacttacagctgaac; G162S, 5′-cattctgtgaacaagacagcttggcttc; S169P, 5′-cattctgtgaacagcttggcttc; D317H, 5′-ctgtgaacagcttggcttc; G162S was identified in a screen for mutants of Aspergillus nidulans (27). Other gene products involved in the same pathway are subunits of the microtubule-based motor protein cytoplasmic dynein, or its regulatory complex dynactin (28–30). LIS1 interacts with subunits of the dynein and dynactin complexes in mammalian cells (25, 31–34). LIS1 also interacts with tubulin (34) and can modulate microtubule dynamics in vitro (35). Furthermore, LIS1 colocalize with NudC, NudEL, mNudE, and γ-tubulin at the centrosome and could control the microtubule nucleation (25, 26, 36). All these results indicate a conserved mechanism from nuclear movement in the fungus and flies to neuronal migration in the brain. An additional LIS1-interacting protein that is functioning in the dynein/dynactin pathway is CLIP-170 (37). We also detected an interaction between LIS1 and doublecortin (DCX) that mutations in the gene product also result in lissencephaly (38).

**Protein Half-life Assay**—293 cells were transfected with LIS1-FLAG-DeRed (wild type or mutations) in 6-well plates using the CaCl2 method. Half-life was measured using pulse-chase as described (42) with some modifications. Prior to addition of [35S]Smethionine, cells were washed and starved. The pulse was for 1 h, followed by addition of an excess of cold methionine. Ten time points were picked (0, 2, 4, 6, 8, 10, 12, 18, and 24 h). The cells were washed and collected in IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100), supplemented with protease inhibitors (Sigma). Immunoprecipitations were performed using M2 beads (Sigma), run on 10% PAGE gels. The gels were fixed, and the signal was enhanced by 30-min incubation with sodium salicylate solution (16%) and PPO, and exposed to phosphoimager film. The signal was quantified using phosphoimager analysis, and the results were plotted. The half-life was deduced from the plotted results.

**Trypsin Cleavage**—LIS1 protein (wild-type and mutations) was translated in a rabbit reticulocyte TntT system (Promega, Madison, WI) with [35S]Smethionine. A portion of the labeled reaction (10 µl) was used for tryptic cleavage as described (11).

**His6-Pull-down Assay**—Recombinant His6 proteins were incubated with transfected 293 cell extract (prepared as described) or rabbit reticulocyte lysate. Pull-down assay was performed as described (38).

**Immunoprecipitation Assay**—Immunoprecipitation was performed in 293 cells using M2 beads (Sigma). Cells were co-transfected with LIS1-FLAG-DeRed (wild-type or mutated) and each of the interacting proteins. Transfected cell extracts were prepared in IP buffer, supplemented with protease inhibitors (Sigma). Each 1-cm plate was washed with PBS, put on ice, and scraped with 1 ml of cold IP buffer. The cells were incubated on ice for 15 min and were vortexed for 10 s every 5 min. After centrifugation at 9000 rpm for 15 min at 4 °C, sample buffer was added and the beads were resuspended. The samples were separated by SDS-PAGE and Western blotted with the corresponding antibody (α-LIS1 monoclonal clone 210, Ref. 35), α-FLAG (M2 clone from Sigma), α-myc (9E10 clone from Sigma), or α-PAF-AH-α1 antibodies (monoclonal clone 9C9; Ref. 43).

**Yeast Two-hybrid**—Yeast transformation and detection of interactions were performed according to manual (OriGene Technologies, Inc.); briefly, three plasmids were transformed into the yeast strain EGY48; pEG-202-bait, pJG4-5-prey and pSH18-34-LacZ (from the DupLEX-A system, OriGene Technologies, Inc.). The baits were LIS1 wild-type or one of the five mutations, and the preys were PAF-AH α1, PAF-AH α2, C, and α-Myc, and NudEL.

The yeast cells were grown to an A600 of 0.6–0.8, harvested at 1500 ± 3 for 3 min, and resuspended in TE (one third of initial volume). The cells were then pelleted again, resuspended in the same volume of TE/LiOAc, and rotated slowly at 30 °C for 2 h. Afterward, the cells were pelleted and resuspended in TE, 0.1 M LiOAc (100 µl transformation). The described bait and prey proteins were incubated with the lacZ fusion of salmon sperm DNA were added to each tube and the cells were incubated for 45 more min. Heat shock was performed at 42 °C for 5 min, and the cells were then spun down, resuspended in TE, and plated on glucose-based plates lacking his, Trp, arg, and aden (H– T– U). Transformed colonies were incubated at 30 °C 2–3 days until colonies appeared. From each interaction 4–5 colonies were streaked on galactose-based plates (SG-H-T-U) containing 1 mg/ml

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2 J. R. De Mey, unpublished data.
LIS1 Mutations

**RESULTS**

LIS1 is an essential protein that controls multiple cellular functions through participation in different protein complexes. Deciphering the precise details of LIS1-protein interaction modes under normal and abnormal conditions may allow us to better understand processes of neuronal migration and brain development. For this analysis the existence of several disease causing amino acid substitutions was utilized.

Our analysis included several assays to determine whether there were global effects of amino acid substitutions; protein folding was tested by limited trypsin cleavage. LIS1 stability was monitored by examining protein degradation, and its intracellular localization was inspected by immunostaining. To resolve whether LIS1 mutations affect specific protein interactions, we first mapped domains of interaction between LIS1 and some of its protein partners. The described protein mutants were then used to test whether they retain the ability to bind these known interacting proteins.

**LIS1 Folding**—It has been shown that multiple WD-containing proteins (including LIS1) retain the β-propeller-like structure similar to the β-subunits of heterotrimeric G-proteins (45). Thus, the WD-containing region is protected when these proteins are subjected to limited proteolytic cleavage. In the following assay, LIS1 carrying the different mutations was expressed in vitro in rabbit reticulocytes and labeled with [35S]methionine. When the lysate was subjected to limited trypsin cleavage, three protected bands were visible in case of the wild type LIS1 protein (Fig. 1), as has been observed before (45). Among the mutants, there were two protected bands in the case of the N-terminal mutation F31S, and three protease protected bands in case of the G162S mutation, whereas the others were almost completely degraded. The results suggest that most of the proteins carrying mutations in the WD domains are misfolded with partial folding of the G162S mutant protein.

**LIS1 Half-life and Intracellular Localization**—Protein half-life was tested by overexpression of LIS1-FLAG-DsRed (wild type and mutated) in human 293 cells. Following transfection protein expression was allowed to proceed for 36 h, and then a pulse of [35S]methionine was added as described under “Experimental Procedures.” Cell lysates were subject to immunoprecipitations followed by separation on SDS-PAGE. The half-life of the proteins was determined from the plotted results (Table I). The results demonstrate that LIS1 is a very stable protein with half-life exceeding 20 h (Table I). LIS1 mutated in F31S and S169P exhibited relatively short half-life, LIS1 mutated in G162S exhibited a longer half-life, whereas LIS1 mutated in D317H and H149R revealed the longest half-life among all five mutations. The LIS1 N-terminal mutation F31S is unique in the sense that it aggregates when overexpressed in HeLa cells (Fig. 2a) and 293 cells (data not shown), although in vitro trypsin cleavage results predicted this protein to be able to retain the WD folding. This phenotype did not change when other LIS1-interacting proteins were coexpressed such as CLIP-170 (Fig. 2, e and f), dynein heavy chain 1, or p50 (data not shown). Interestingly, when this mutant protein was coexpressed with the α1 PAF-AH subunit, NuDε, or NuDεL, the typical aggregates were less observed. Coexpression with the α1 PAF-AH subunit solubilized most of the aggregates (visible in Fig. 2, c and d) but did not rescue its localization. In mitotic cells, no mutant LIS1 was observed at the spindle poles (Fig. 2, l and Fig. 3, m–p and t–x). When NuDεL (Fig. 2, g and h) or NuDε (data not shown) was coexpressed, DsRed-F31S was expressed as small punctate dots that colocalized with either binding partner. In mitotic cells, DsRed-F31S localized to the spindle poles and kinetochores (Fig. 2, i–k). Because the intracellular localization was affected by coexpression with the α1 PAF-AH subunit or by NuDε or NuDεL, we tested the possible effect on the mutant protein half-life (Table I). Interestingly, the coexpression with the α1 PAF-AH subunit increased significantly protein stability, whereas coexpression with NuDεL had no such effect. Therefore, specific localization is not necessarily correlated with protein stability. Next, we tested whether overexpression of the α1 PAF-AH subunit or by NuDεL had any observable effect upon the intracellular localization of all the other mutants, and no effect was observed (data not shown). Because the trypsin cleavage assay suggested partial folding of G162S, we tested whether overexpression of the α1 PAF-AH subunit or NuDεL may affect the half-life of this mutant protein (Table I). No such effect was observed.

All overexpressed mutated LIS1 forms did not visibly localize to the normal LIS1 intracellular sites. These mislocalizations are mostly prominent in structures related to dynein/dynactin or CLIP-170, as microtubule tips, spindles, centrosome, kinetochore, and some of its protein partners. The described protein mutations, we first mapped domains of interaction between LIS1 and the different WD peptides) translated in the rabbit reticulocyte TnT system (Promega) labeled with [35S]methionine. The lysate was then subjected to limited trypsin cleavage. It can be noted that the N-terminal mutation F31S, and the G162S mutant proteins were partially protected, whereas the others were completely degraded.

**X-gal and incubated overnight at 30 °C until the appearance of a blue color.**

**WD Binding to Microtubules**—Phosphocellulose-purified tubulin was assembled into microtubules with addition of Taxol® (pachitaxel; final concentration 30 μM) or nocodazole (10 μg/ml) as described (44). The assembled microtubules were mixed with 10 μl of [35S]methionine-labeled proteins (LIS1 and the different WD peptides) translated in the rabbit reticulocyte TnT system (Promega). The mixtures were incubated at 37 °C for 10 min and separated into two fractions as described (44). The insoluble fractions (pellets) of each experiment were run on 15% SDS-PAGE gels and stained with Coomassie Brilliant Blue (Bio-Rad). After destaining the gels were washed with H2O for 20 min and the signal was enhanced by 30-min incubation with sodium salicylate solution (16%) at room temperature.

**HEK 293 cells were transfected with LIS1-FLAG-DeRed (wild type and mutated versions) and the proteins were expressed for 36 h. A pulse of [35S]methionine was added as described under “Experimental Procedures.” Cell lysates were subject to immunoprecipitations followed by separation on SDS-PAGE. The half-life of the proteins was determined from the plotted results (Table I).**

### TABLE I

| Protein half-life | Grade |
|------------------|-------|
| **Grade**<sup>a</sup> | **Half-life** |
| LIS1 WT | >20 h |
| F31S 4a1 | 1.7 ± 0.3 |
| H149R 3a | 10.5 ± 3.5 |
| G162S 4a2 | 5.2 ± 0.7 |
| S169P 6a | 2.13 ± 0.7 |
| D317H 4a1 | 7.8 ± 2.1 |
| F31S + α1 NA | 6.78 ± 0.52 |
| F31S + NuDεL NA | 0.87 ± 0.002 |
| G162S + α1 NA | 7.5 ± 0.2 |
| G162S + NuDεL NA | 4.1 ± 1.6 |

* LIS grade according to Leventer et al. (15).
Fig. 2. Localization of LIS1(F31S)-FLAG-DsRed mutant protein in HeLa cells. HeLa cells were transiently transfected with plasmids encoding LIS1(F31S)-FLAG-DsRed (a and b), or transiently co-transfected with plasmids encoding LIS1(F31S)-FLAG-DsRed and GFP-α1 (c and d, l–n), or transiently co-transfected with plasmids encoding LIS1(F31S)-FLAG-DsRed and CLIP-170 (e and f), or transiently co-transfected with plasmids encoding LIS1(F31S)-FLAG-DsRed and NudEL (g, h) or mNudC (i–k). Panels a, c, e, g, i, and l show the DsRed signal. Panels b, d, f, h, j, and m show the GFP or fluorescein isothiocyanate signal. Panels k and n show the DAPI signal. Bar, 10 μm.

LIS1 Mutations

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Fig. 3. Localization of LIS1 mutant proteins in HeLa cells. HeLa cells were transiently transfected with plasmids encoding LIS1-FLAG-DsRed (a, i, and q), LIS1(D317H)-FLAG-DsRed (e and m) or LIS1(G162S)-FLAG-DsRed (u). The cells were labeled with the monoclonal anti-EB1 antibody (b, f, j, and n) or with the anti-CLIP-170 TA antibody (r and v). Panels c, g, k, o, s, and w are superimpositions of...
LIS1 Mutations

Interactions of LIS1 domains with different protein partners

The WD domains, N-terminal, and full-length LIS1 were translated in reticulocyte system in the presence of [35S]methionine. The labeled fragments were then subjected to pull-down experiments with recombinant NudC, NudE, NudEL, αL2, and LIS1. NudE and NudEL interact mainly with the full-length protein, and no region seems to be crucial for the interaction. In the case of NudC, on the other hand, WD5 and WD7 are involved in the interaction and bind even stronger then the whole protein. PAF-AH catalytic subunits α1 and α2 interact weakly with LIS1 via WD2, WD3, and WD7, whereas dimerization is mediated through WD3, WD6, and the N-terminal region. The regions that appear more important for interactions are marked in bold.

| LIS1 | WD1 | WD2 | WD3 | WD4 | WD5 | WD6 | WD7 | N-terminal |
|------|-----|-----|-----|-----|-----|-----|-----|------------|
| NudC | 13  | 19  | 6   | 10  | 15  | 32  | 16  | 35         | 12         |
| NudE | 54  | 4   | 2   | 7   | 4   | 4   | 3   | 0          | 0          |
| NudEL| 81  | 8   | 2   | 7   | 3.5 | 4   | 3.5 | 3          | 0          |
| αL2  | 16  | 15  | 18  | 17  | 0   | 12  | 12  | 18         | 10         |
| LIS1 | 87  | 25  | 16  | 60  | 17.7| 6.9 | 5.4 | 34.3       | 2.8        |
| DCX  | 78  | 6.5 | 6.8 | 6   | 18.5| 9.3 | 6.8 | 2.4        | 3.2        |
| CLIP  | 82  | 2.8 | 11.6| 5.9 |     |     |     |            |            |

TABLE II

LIS1 Mutations

Interactions of LIS1 domains with different protein partners

The WD domains, N-terminal, and full-length LIS1 were translated in reticulocyte system in the presence of [35S]methionine. The labeled fragments were then subjected to pull-down experiments with recombinant NudC, NudE, NudEL, αL2, and LIS1. NudE and NudEL interact mainly with the full-length protein, and no region seems to be crucial for the interaction. In the case of NudC, on the other hand, WD5 and WD7 are involved in the interaction and bind even stronger then the whole protein. PAF-AH catalytic subunits α1 and α2 interact weakly with LIS1 via WD2, WD3, and WD7, whereas dimerization is mediated through WD3, WD6, and the N-terminal region. The regions that appear more important for interactions are marked in bold.

| LIS1 | WD1 | WD2 | WD3 | WD4 | WD5 | WD6 | WD7 | N-terminal |
|------|-----|-----|-----|-----|-----|-----|-----|------------|
| NudC | 13  | 19  | 6   | 10  | 15  | 32  | 16  | 35         | 12         |
| NudE | 54  | 4   | 2   | 7   | 4   | 4   | 3   | 0          | 0          |
| NudEL| 81  | 8   | 2   | 7   | 3.5 | 4   | 3.5 | 3          | 0          |
| αL2  | 16  | 15  | 18  | 17  | 0   | 12  | 12  | 18         | 10         |
| LIS1 | 87  | 25  | 16  | 60  | 17.7| 6.9 | 5.4 | 34.3       | 2.8        |
| DCX  | 78  | 6.5 | 6.8 | 6   | 18.5| 9.3 | 6.8 | 2.4        | 3.2        |
| CLIP  | 82  | 2.8 | 11.6| 5.9 |     |     |     |            |            |

* Ref. 38.

** Ref. 37.

not capable of competing with mammalian LIS1 over dimerization; hence, this system is the only one where endogenous LIS1 plays no role.

Co-immunoprecipitation—For testing the possible interac-

LIS1 is mediated via WD3, WD6, and the N-terminal region (the importance of the N-terminal region for dimerization has been reported by us previously (Ref. 19)).

The domain important for interaction with the microtubule cytoskeleton was determined by binding of the different WDs to preassembled microtubules (Fig. 4b). Paclitaxel was used to stabilize microtubules, and a control nocodazole was used to block polymerization. The mixture was then incubated with the [35S]methionine-labeled domains and centrifuged to create soluble and insoluble fractions. In Fig. 4b only the insoluble fractions treated with paclitaxel or nocodazole are shown. The paclitaxel-insoluble fraction will be enriched for proteins that bind microtubules. Tubulin, of course, can be found in this fraction, whereas it is depleted from the nocodazole control pellet (panel b, lower gel). Some of the labeled proteins are still found in the pellet; however, this fraction may represent unfolded proteins that aggregates and pellets in a nonspecific way. By subtracting the nocodazole-treated fraction from the paclitaxel-treated one, it can be determined whether a protein is specifically bound to the microtubule cytoskeleton. Thus, the WD domains that are indicated as involved in microtubule binding are WD2, WD5, WD6, and WD7, and the N-terminal domain (Fig. 4b). Remarkably, all the patients’ mutations are found in domains that are involved in binding to microtubules (the N terminus, WD2, and WD5). This result is consistent with the immunostaining data, which show a marked reduction in the localization of mutated LIS1 in cellular structures that are associated with microtubules (Figs. 2 and 3).

Yeast Two-hybrid—LIS1 (wild type and mutated) was expressed as a bait protein in yeast cells together with lacZ reporter and the preys, NudEL, CLIP-170 (the C-terminal region), and αL2. In this system CLIP-170 interacts only with the normal LIS, and no obvious interaction is detected with any of the mutations (Fig. 5, left panel). α1 and α2 (only α1 shown) interact with normal LIS1 and much more weakly with the mutations F31S and G162S (middle panel). NudEL interacts with normal LIS1 but also with the mutated LIS1 F31S and G162S with the same strength (right panel). It is possible that other proteins found in the yeast cell influence the final structure of these two mutated LIS1 forms enabling the binding to the α subunits (to some degree) and to NudEL. It is worth mentioning that we have found that the yeast LIS1 (PAC1p) is

LIS1-FLAG-DsRed (red) and EB1 or CLIP-170 (green) signals. Panels d, h, l, p, t, and x correspond to DAPI signal. The insets show the co-localization of LIS1-FLAG-DsRed and EB1 on microtubule tips. As expected, LIS1-FLAG-DsRed is localized on centrosomes (arrows) and microtubule tips in prophase (a–d), on spindle poles (see arrows) and kinetochores in prometaphase (q–t, arrows), and on cortical sites in late prometaphase (q–t, arrows). Unlike wild type LIS1, mutant proteins do not localize on centrosomes (e–h, arrows), microtubule tips (e–h), kinetochores, spindle poles (m–p and n–x), and cortical sites (u–x, arrows). All images are maximal-intensity projections of z/x optical section stacks, acquired by three-dimensional deconvolution microscopy. Bars, 5 μm.

FIG. 4. a, schematic presentation of mutations. LIS1 domains with the position of the mutations are shown schematically. LIS1 is composed of an N-terminal region of 95 amino acids that includes a Lis-H (LIS1 homology domain amino acids 8–37) and a coiled-coil domain (amino acids 51–79) important for dimerization. b, interactions of LIS1 domains with microtubules. Binding to preassembled tubulin was performed as described. The regions that are involved in the interaction are WD2, WD5, WD6, WD7 and the N-terminal region. n = nocodazole-treated, T = paclitaxel-treated, T-n = nocodazole-treated subtracted from paclitaxel-treated. The experiment was repeated three times, and a representing gel was picked.

3 M. Caspi, unpublished data.
We tested five different missense mutations for their gross biochemical properties as well as their ability to retain interactions with known protein partners. A summary of the experimental results is presented in Table III. Clearly, no single assay can point out to the predicted lissencephaly severity.

Mutation H149R causes the most severe phenotype among the lissencephaly patients analyzed in this study. The biochemical profile of the mutated protein can provide the explanation. The protein was completely degraded in the trypsin assay indicating that its folding was most likely incorrect. The protein stability was intermediate. Combined with the summation of all interaction experiments, it is clear that the mutated protein can barely function. In addition, this mutation was examined previously demonstrating instability, misfolding (11), or abolishment of protein interactions (36, 47, 48). It is not surprising that the substitution of residue 149 from histidine to arginine resulted in such a devastating malformation. The biochemical profile of mutation S169P was poorer by our criteria than the one seen in mutation H149R. Mutation S169P is the only substitution that is in clear contrast to the observed phenotype. The mutated protein is folded incorrectly, and its stability is extremely reduced. The interaction data are the same as for mutation D317H, which results in a much more severe phenotype. This mutation has also been investigated in relation to its interaction with PAF-AH subunits (47), NudEL (48), and NudE (36), and no interactions were observed using yeast two-hybrid system (48). These results can only be settled if we accept the biochemical predictions (14) and the authors’ (14) notion that the patient is a mosaic. In such a case the severely affected protein (substitution of residue 169 from serine to proline) is expressed in only some of the cell population of the brain. In other cells the expression of wild type LIS1 protein can diminish the resulted malformation. Two of the mutations, F31S and D317H, cause the same lissencephalic grade (4α(1)). However, looking closely at the experimental data reveals different biochemical properties. Mutation F31S resides in the N-terminal region of LIS1 protein (Fig. 4a) within the recently described LIS-H domain (18). The N-terminal region is predicted to remain external to the β-propeller structure characterized of WD domains. The mutated protein was expected to be partially protected from trypsin cleavage as the globular WD domain is most likely still intact. However, the F31S mutation severely affects protein stability, and interaction abilities are reduced. The aggregates that are formed in cells may suggest nonfunctional mutated protein. A possible hypothesis that may explain variable phenotypes is that a LIS1 partner could be up-regulated in some patients to provide conformational support or stability to a mutated protein. Indeed, we demonstrated that the interactions with the PAF-AH catalytic subunit, NudE, or NudEL affected the F31S aggregates. Nevertheless, the effect on stability differed between the two coexpressed proteins. Although overexpression of the PAF-AH catalytic subunit increased the half-life of the mutated protein, overexpression of NudEL did not. It might suggest that LIS1 stability and cellular localizations are maintained by two different mechanisms.

The biochemical profile of LIS1 carrying the D317H mutation is somewhat different. Mutation D317H is located in WD5 (Fig. 4a), which is unique among the WD repeats of LIS1 as it contains a hinge region, rich in serine residues. The mutated protein is most likely improperly folded, although the half-life assay demonstrated that it retained some of its stability. The interaction information reveals a probable defect that can obstruct the binding of other proteins. Interestingly, the main observable difference between these mutations resides in the ability to interact in the yeast system. As mentioned before in

**DISCUSSION**

The identification of variable lissencephaly phenotypes has opened a wide field for investigation of neuronal migration disorders. The fact that a single amino acid substitution can result in different disease manifestation may help us to address the complicated task of elucidating the mechanisms behind brain development. Indeed, the biochemical outcomes of these mutations reflect a variety of possibilities as to how mutated LIS1 may function in a cellular context.
In this system, the endogenous yeast LIS1 has little or no effect on the mutated LIS1 interactions. Maybe binding abilities are more severely defected in the case of D317H; thus, it is unable to bind any known partner without the presence of a normal LIS1. Evidently, the two mutated proteins behave in a different biochemical and cellular context. But, each of the two separate pathways eventually leads to the same lissencephaly phenotype. Mutation G162S located in the WD2 repeat of LIS1 man-

**Fig. 6. Co-immunoprecipitations.** LIS1-FLAG-DsRed wild type or mutated were expressed in 293 cells alone (a and c) or together with each of the tested partners (b, d, e, f, and g). Co-immunoprecipitation was done with α-FLAG antibodies (a, b, d, e, f, and g) or with α-LIS1 antibody (polyclonal) (c). a, endogenous LIS1 was tested for dimerization with the transfected LIS1 (wild type and mutants). b, the interactions between GFP-α1 and the different LIS1 forms were examined as described. c, endogenous NudC was used to check the interactions with the different LIS1 forms. d, a portion of the dynein heavy chain fused to a Myc tag was used to assay its interaction with LIS1-FLAG-DsRed wild type or mutated. e and f, Myc-tagged NudE and NudEL were co-transfected together with the different LIS1 forms to test the interactions with them. g, the C-terminal portion of CLIP-170 fused to GFP was used to examine its interaction with LIS1-FLAG-DsRed wild type or mutated.
ifests a rather mild phenotype exhibited by the patient. This outcome is supported by the biochemical data. The protein is partially folded and is relatively stable. Furthermore, interactions with other proteins were only slightly affected. Overall, the results presented here are consistent with the weak manifestation of lissencephaly syndrome.

The discovery of different disease phenotypes within lissencephaly patients opens a window to new insights regarding the processes in which the human brain is evolving. By investigating the mutated proteins, we have gained one step further in understanding these processes and elucidating their meaning.

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TABLE III
Summary of LIS1 mutation properties

| Folding          | Interactions | Stability   | Grade |
|------------------|--------------|------------|-------|
| L1S1             | Normal       | Normal     | Very Stable | WT |
| F31S             | Partial      | Partial    | Unstable   | 4a |
| H149R            | Abnormal     | Reduced    | Medium     | 3a |
| G162S            | Partial      | Partial    | Intermediate | 4a2 |
| S169P            | Abnormal     | Reduced    | Unstable   | 6a |
| D317H            | Abnormal     | Reduced    | Medium     | 4a1 |

LIS1 Mutations
Experimental results of the properties of mutated LIS1 proteins are listed in a schematic fashion. Interactions include data from localization studies, immunoprecipitations, and yeast two-hybrid.

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