Reduction of microtubule catastrophe events by LIS1, platelet-activating factor acetylhydrolase subunit

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Forming the structure of the human brain involves extensive neuronal migration, a process dependent on cytoskeletal rearrangement. Neuronal migration is believed to be disrupted in patients exhibiting the developmental brain malformation lissencephaly. Previous studies have shown that LIS1, the defective gene found in patients with lissencephaly, is a subunit of the platelet-activating factor acetylhydrolase. Our results indicated that LIS1 has an additional function. By interacting with tubulin it suppresses microtubule dynamics. We detected LIS1 interaction with microtubules by immunostaining and co-assembly. LIS1–tubulin interactions were assayed by co-immunoprecipitation and by surface plasmon resonance changes. Microtubule dynamic measurements *in vitro* indicated that physiological concentrations of LIS1 indeed reduced microtubule catastrophe events, thereby resulting in a net increase in the maximum length of the microtubules. Furthermore, the LIS1 protein concentration in the brain, measured by quantitative Western blots, is high and is approximately one-fifth of the concentration of brain tubulin. Our new findings show that LIS1 is a protein exhibiting several cellular interactions, and the interaction with the cytoskeleton may prove to be the mode of transducing a signal generated by platelet-activating factor. We postulate that the LIS1–cytoskeletal interaction is important for neuronal migration, a process that is defective in lissencephaly patients.

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(Mizuguchi *et al.*, 1995). Independently, LIS1 was cloned and identified as a subunit of intracellular platelet-activating factor acetylhydrolase [PAF-AH(Ib)] (Hattori *et al.*, 1994). Although associated with the enzyme, the LIS1 subunit does not contain any catalytic activity. LIS1 (Reiner *et al.*, 1995), as well as the two other subunits of PAF-AH (Albrecht *et al.*, 1996) are expressed in brain regions undergoing active proliferation, differentiation, and migration. Platelet-activating factor [PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine] is a potent phospholipid messenger molecule, implicated in a variety of physiological events (Hanahan, 1986; Bazan *et al.*, 1993), including neural development (Kornecki *et al.*, 1988) and function (Bito *et al.*, 1992; Kato *et al.*, 1994). The intra- and extracellular levels of PAF are well controlled by a specific group of phospholipases, PAF-acetylhydrolases (PAF-AHs), which inactivate PAF by hydrolyzing the *sn*-2-acetyl group that produces inactive lyso-PAF. LIS1, the β -subunit of the enzyme contains seven WD repeats (Reiner *et al.*, 1993) similar to the β -subunit of the heterotrimeric G proteins (WD repeats reviewed in Neer *et al.*, 1994). Recently, the structure of the α 1 catalytic subunit was solved and found to closely resemble that of p21^{ras} and other GTPases (Ho *et al.*, 1997). Thus the intact PAF-AH(Ib) molecule is a unique G-protein-like (α 1/ α 2) β trimer. Proteins containing WD repeat motifs are very diverse in function. Several members of this protein family form complexes with other proteins, therefore possibly implying that LIS1 may participate in several protein–protein interactions. This hypothesis led us to investigate interactions between LIS1 and additional cellular proteins. In our work, we have identified a new interaction between LIS1 and tubulin. This interaction influenced microtubule dynamics *in vitro*. Our new findings show that LIS1 is a protein exhibiting several cellular interactions, and the interaction with the cytoskeleton may prove to be the mode of transducing a signal generated by platelet-activating factor. We postulate that the LIS1–cytoskeletal interaction is important for neuronal migration, a process that is defective in lissencephaly patients.

Introduction

Our previous work has described the involvement of the LIS1 gene in Miller–Dieker lissencephaly (Reiner *et al.*, 1993). This finding was recently strengthened by the identification of point mutations and an intragenic deletion in the LIS1 gene in isolated lissencephaly patients (Nigro *et al.*, 1997). Lissencephaly is a human brain malformation characterized by a smooth cerebral surface and a disordered organization of the cortical layers resulting from a defect in neuronal migration (Barth, 1987; Aicardi, 1989). Indeed, the protein product of the LIS1 gene was found to be reduced in brain sections from lissencephaly patients

Results

LIS1 interaction with microtubules

We used anti-LIS1 antibodies to immunostain human melanoblastoma cells and rat primary neurons. The observed fibrous structures (Figure 1A, E and G) resembled immunostained microtubule (MT) cytoskeleton (Figure 1B and F). Similar immunostained structures were observed in mouse fibroblasts (NIH 3T3), human cervical carcinoma (HeLa) and monkey kidney (COS-M6) cells (data not shown). Significantly, LIS1 was distributed along axons and dendrites within neurons (Figure 1E and G). Furthermore, treating melanoblastoma cells with the MT-

Fig. 1. Co-localization of LIS1 and microtubules. Melanoblastoma cells (ATCC HTB 68) were permeabilized with Triton X-100 (0.5%) and fixed with 3% paraformaldehyde. The cells were co-stained with polyclonal affinity-purified anti-LIS1 antibodies (A and C) and monoclonal anti-tubulin antibodies (B and D) and visualized by a fluorescently labeled second antibody. Fibrous structures were significantly reduced after treatment with nocodazole (C and D). Rat primary neurons were fixed with 4% paraformaldehyde, permeabilized with Triton X-100 (0.25%) and the cells co-stained with polyclonal affinity purified anti-LIS1 antibodies (E and G) and monoclonal anti-tubulin antibodies (F) or monoclonal anti-MAP2 (H).

disrupting drug, nocodazole, resulted in reduced immuno-reactivity of both LIS1 (Figure 1C) and tubulin (Figure 1D).

These initial *in vivo* data were further corroborated biochemically. When tubulin was purified on a phosphocellulose column [after multiple cycles of assembly–disassembly (Williams, 1992)], LIS1 was co-eluted with other microtubule-associated proteins (MAPs) (data not shown). Furthermore, LIS1 coassembled with MTs *in vitro* (Figure 2). Brain extract containing tubulin was polymerized to MTs, the supernatant preserved, and MTs pellets washed with salt to dissociate conventional MAPs. The assembly process was monitored by reacting the blot with anti-tubulin and anti-MAP2 antibodies (Figure 2, right panel). The same blot was reacted with monoclonal anti-LIS1 antibodies (Figure 2, left panel). LIS1 was found in all the fractions but was enriched in the MAP fraction (Figure 2, lane 3, left panel) and was in reduced levels in the MT-depleted fraction (Figure 2, lane 4, left panel). In order to probe the specificity of LIS1 interaction with polymerizing microtubules, we have coassembled *in vitro* translated LIS1 with tubulin (Table 1). Correct folding of the *in vitro* translated LIS1 has been demonstrated previously (Garcia-Higuera *et al.*, 1996). The assembly efficiency was determined by monitoring tubulin levels in the supernatant and in the pellet after sedimentation of the polymerized microtubules (an example of such an experiment is shown in Figure 3). In the absence of tubulin, LIS1 was barely detectable in the pellet. Most of

Fig. 2. Endogenous LIS1 coassembles with microtubules. Left panel: immunoblot using monoclonal anti-LIS1 antibody (clone 338.40). Right panel: same blot reacted with anti-tubulin and anti-MAP2 antibodies, monitoring efficiency of assembly and dissociation, respectively. Equal amounts of protein samples (40 µg) were resolved on 10% SDS-PAGE. Microtubule assembly was induced from bovine brain extracts by adding GTP and taxol. The microtubules were isolated by low speed centrifugation. MAPs were dissociated from microtubules by using 0.4 M NaCl. Crude bovine brain extract (lane 1), microtubule pellet after 0.4 M NaCl wash (lane 2), MAP fraction (lane 3). Note the LIS1 enrichment in lane 3 on the left panel and MAP2 on the right panel. Unassembled supernatant after the assembly reaction contained some LIS1 on the left panel, but no tubulin on the right panel (lane 4).

the tubulin (86%) is found in the supernatant without the addition of microtubule stabilizing agent. In concordance 18% of the *in vitro* translated full-length LIS1 protein is detected in the microtubule pellet. The addition of the drug taxol that stabilizes microtubules resulted in the precipitation of most of the tubulin (67%) and of 50% of

Fig. 3. *In vitro* translated LIS1 coassembles with microtubules. (A) *In vitro* translated ^{35}S -labeled LIS1, the full-length protein and the short form of LIS1 (indicated by an arrow) were subject to microtubules cosedimentation assay as described. Samples were incubated with (+) or without (-) tubulin, allowing the tubulin to polymerize. Nocodazole or taxol were added to samples as indicated. After centrifugation, supernatant (S) and pellet (P) fractions were analyzed on 10% SDS-PAGE gels. The gels were stained with Coomassie blue (upper panel) dried and exposed to X-ray film (lower panel). (B) The table represents an average of three experiments ($\pm$ standard deviation). The levels of tubulin were measured by densitometry and the levels of *in vitro* translation products in the pellet fraction were quantified by phosphorimager.

full-length LIS1. The addition of specific monoclonal anti-LIS1 antibodies competed with the interaction of LIS1 with microtubules and reduced LIS1 binding to microtubules by 16%. Addition of 0.4 M NaCl completely abolished the LIS1-microtubule interaction (5%). This ionic strength of NaCl is known to interfere with the interaction of MAPs and microtubules (Vallee, 1986). The microtubule destabilizing drug nocodazole resulted in the transfer of both tubulin and LIS1 to the supernatant fraction. In addition to the full-length form, internal initiation of the protein (at the second methionine) results in a shorter form (32 kDa) (Garcia-Higuera *et al.*, 1996). The shorter form of LIS1 has a native molecular weight in close proximity to that estimated from its primary sequence, indicating correct folding (Garcia-Higuera *et al.*, 1996). As depicted in our experiments, this short form of LIS1 does not interact well with the microtubules. The interaction of the short form with taxol-stabilized microtubules is only half of that of the full-length protein (23% versus 50%). This result suggests that amino acids located in the N-terminal region are important for LIS1-microtubule interaction.

LIS1 interaction with tubulin

Conventional MAPs can be classified into two categories, structural MAPs and motility related MAPs (motor proteins). In addition to these conventional MAPs several tubulin-binding proteins (TBP or unconventional MAPs) have been identified in recent years serving different cellular functions in addition to, or in combination with, their tubulin binding properties. One example is elongation factor 1 α (Efl α) (Shiina *et al.*, 1994; Condeelis, 1995). Efl α is an essential component of the eukaryotic translational apparatus but this polypeptide also plays a role as regulator of cytoskeletal rearrangement. Efl α activity caused severing of stable microtubules, demonstrated both *in vitro* and *in vivo* by microinjection into fibroblasts. A second example is oncoprotein 18, which was found to interact with tubulin dimers and increase the catastrophe

Fig. 4. Co-immunoprecipitation of ^{35}S -labeled LIS1 and α tubulin. *In vitro* translated LIS1 was incubated with (+) or without (-) phosphocellulose purified tubulin. Note that LIS1 precipitated only in the presence of both tubulin and anti- α tubulin antibodies.

rate of microtubules (Belmont *et al.*, 1996). Therefore, after detecting LIS1-MT interactions, we proceeded to investigate whether LIS1 can interact with tubulin subunits in addition to its interaction with microtubules. This interaction was initially investigated by co-immunoprecipitation (Figure 4). *In vitro* translated LIS1 precipitated only in the presence of tubulin and anti-tubulin antibodies. In order to look at finer specifications of this interaction we used the BIAcoreTM, a system that measures changes in surface plasmon resonance that are proportional to the changes in the mass of molecular species bound to the surface (Fagerstam *et al.*, 1992; Jonsson *et al.*, 1992; Malmqvist, 1993; Raghvan *et al.*, 1995). The mass differences measured reflect the kinetic events including association and dissociation of complexes that are composed of bound molecules and other molecules brought into contact with them in the flow cell. The response of LIS1 to tubulin

Fig. 5. LIS1–tubulin interaction using surface plasmon resonance change analysis. **(A)** Binding of tubulin dimers to immobilized LIS1 is specific. LIS1 was immobilized to the sensor chip at two densities (channel 3, 3000 RU and channel 4, 6000 RU). To differentiate the specific binding signal from bulk refractive index changes, a blank channel (1) was prepared by a flow buffer instead of a protein solution during the immobilization phase. As a nonspecific control, BSA was immobilized as well (5000 RU, channel 2). The sensogram shows that tubulin did not bind to BSA nor to the blank channel, and that the specific signal increased when more LIS1 was immobilized to the surface. Trace 1, blank channel; Trace 2, 5000 RU BSA; Trace 3, 3000 RU LIS1; Trace 4, 6000 RU LIS1. **(B)** Signal amplitude is proportional to the analyte concentration. Different tubulin concentrations ranging from 1 to 1.8 μ M were introduced to the immobilized LIS1 channel (2000 RU) at a constant flow rate of 10 μ l/min; 1, 1.8 μ M; 2, 1.6 μ M; 3, 1.4 μ M; 4, 1.2 μ M; 5, 1 μ M.

is specific and dose-dependent; binding of tubulin to immobilized LIS1 is directly proportional to the amount of bound protein (ligand levels, Figure 5A), and to the concentration of tubulin (analyte level, Fig 5B).

LIS1 reduces catastrophe events

The interaction with the tubulin subunits indicated that LIS1 may directly affect the parameters of microtubule dynamics (Mitchison *et al.*, 1984a,b). Individual MTs display dynamic instability with transitions between growing and shortening phases (Horio *et al.*, 1986). The transition from the growth to the shortening phase is known as a catastrophe event. Here we measured the length of individual MTs by differential interference contrast (DIC) microscopy, combined with video enhancing and image analysis. Our results indicated that MT dynamics are suppressed by substoichiometric concentrations of LIS1. Adding one subunit of LIS1 to 15 or five subunits of tubulin dimers reduced the frequency of catastrophe events and increased the maximum length of MTs very significantly. The velocity of both growth and shortening phases were, however, unaffected (Figure 6). Changes in maximal length could arise from changes in growing and shortening velocities or by changes in catastrophe and rescue (the transition to growing state) frequencies. Our results can not rule out LIS1 effect on rescue frequency in addition to the frequency of catastrophe events. In order to determine the physiological ratio of LIS1 to tubulin, the concentrations of both molecules were measured by quantitative Western blot in bovine brain. Tubulin was found to comprise $2.15 \pm 0.15\%$ of the total protein (using a monoclonal antibody). These results are very close to the results detected by Drubin *et al.* (1988), where the level of tubulin was measured in PC12 cells. LIS1 comprised $0.34 \pm 0.05\%$ of the total protein (using a polyclonal antibody), or $0.56 \pm 0.11\%$ (using a monoclonal antibody). Therefore, the physiological ratio between these proteins is one

Fig. 6. LIS1 affects MT dynamics. DIC microscopy was used to measure the dynamics of MTs polymerizing from axoneme nucleation sites. The growing and shortening dynamics of plus ends were analyzed as described in Materials and methods. LIS1 was added to a constant concentration of 10 μ M tubulin, and four ratios of LIS1/tubulin were used: 0 (no LIS1), 1:100, 1:15 and 1:5. No significant changes were found in the growing and shortening velocities (lower panel). However, both the catastrophe frequency and the maximum length of MTs with a LIS1/tubulin ratio of 1:15 and 1:5 changed significantly (P values are indicated) (top panel).

subunit of LIS1 per six or four subunits of tubulin dimers. Thus the ratio of one subunit of LIS1 per five subunits of tubulin used in these experiments was within the measured physiological range.

Discussion

LIS1 is a multi-faceted protein that was previously identified as a subunit of PAF-acetylhydrolase (Hattori *et al.*, 1994). Our results demonstrate that LIS1 binds tightly to microtubules and tubulin in *in vitro* assays, yet the

immunohistochemistry of LIS1 distribution in fixed cells shows somewhat punctate distribution along microtubules in melanoblastoma cells (Figure 1). We assume that only a portion of LIS1 is found in association with microtubules and that some is in association as a subunit of PAF-AH and these two interactions do not occur simultaneously. This assumption is based on undetectable enzymatic activity in microtubule preparations (O.Reiner, O.Schmuel and T.Sapir, unpublished results), retention of PAF-AH activity in the soluble fractions, and the higher concentration of LIS1 in comparison with the $\alpha 1$ and $\alpha 2$ PAF-AH subunits (O.Reiner, O.Schmuel and T.Sapir, unpublished results). Our results show that the new interactions of LIS1 with MTs and tubulin directly influence MT dynamics. The catastrophe rate frequency of MTs is reduced with lower than physiological concentrations and typical physiological concentrations of LIS1 (e.g. one subunit of LIS1 per 15 or five subunits of tubulin), leading to a concomitant increase in the maximum length of MTs. These results suggest that even if only a portion of LIS1 is associated with tubulin, this interaction may affect microtubule dynamics. This finding implies that LIS1 may be one of the factors that regulates microtubule dynamics during neuronal differentiation and migration. The dynamic instability of MTs may be an important factor for their rapid growth response to various environmental signals. Neurons contain a dense array of MTs suggesting a significant contribution of this cytoskeletal element to the architecture and the function of these highly specialized cells.

Classical MAPs can be classified into two categories: structural MAPs and motility related MAPs (motor proteins). Classical MAPs can affect MT dynamics (recently reviewed in Maccioni *et al.*, 1995; Mandelkow *et al.*, 1995). This was demonstrated in results obtained with a MAP, tau, that stimulated nucleation, reduced the subunit critical concentration, increased the rate of MT elongation and decreased catastrophe frequency (Cleveland *et al.*, 1977a,b; Bre *et al.*, 1990). Tau suppressed the steady-state MT dynamics at tau:tubulin ratios of 1:175 and 1:15 (Panda *et al.*, 1995). These ratios spanned those found in undifferentiated and differentiated PC12 cells (Drubin *et al.*, 1988). Adding these concentrations of tau to MTs did not result in any detectable change in MT mass. It is well known that drugs that decrease the dynamic behavior of MTs inhibit neurite extension (Letourneau *et al.*, 1984; Tanaka *et al.*, 1995). Thus, growth cone advance and the rate of neurite elongation relies on the proper control of assembly and disassembly of MTs. Can mutations in MAPS result in severe neuronal abnormalities? Gene targeting of tau resulted in a subtle phenotype (Harada *et al.*, 1994). The nervous system of tau-deficient mice appeared to be immunohistologically normal. However, in some small-calibre axons, microtubule stability was decreased and microtubule organization was significantly changed. It has been suggested that an observed increase in MAP 1A may have compensated for the functions of tau in large-calibre axons. Recently, mice containing a mutation in a different MAP, MAP 1B, were described (Edelmann *et al.*, 1996). Mice that were homozygous for the modification died during embryogenesis. The heterozygotes exhibited a spectrum of phenotypes including slower growth rates, lack of visual acuity in one or

both eyes, and motor system abnormalities. Histochemical analysis of the severely affected mice revealed that their Purkinje cell dendritic processes are abnormal, do not react with MAP1B antibodies and show reduced staining with MAP1A antibodies. Similar changes were observed in the olfactory bulb, hippocampus and retina, providing a basis for the observed phenotypes. Based on these two mutations it is clear that impairment of MAP functions may result in a neurological phenotype and the severity is dependent upon the locus involved.

In recent years several unconventional MAPs have been identified as serving cellular functions in addition to or in combination with their tubulin binding properties (Kapeller *et al.*, 1993; Lehrich and Forrest, 1994; Shiina *et al.*, 1994; Kiley and Parker, 1995; Reszka *et al.*, 1995). Elongation factor 1 α (Ef1 α) (Shiina *et al.*, 1994; Condeelis, 1995), an essential component of the eukaryotic translational apparatus, also plays a role as a regulator of cytoskeletal rearrangement. Ef1 α activity caused severing of stable microtubules, demonstrated both *in vitro* and *in vivo* by microinjection into fibroblasts. Several unconventional MAPs are known components of signal transduction pathways. The protein kinase C isozyme (Kiley and Parker, 1995) PKC-zeta, colocalizes with MTs both in interphase and metaphase cells (Lehrich and Forrest, 1994). The p85 subunit of phosphatidylinositol (PI)-3' kinase, which catalyses the formation of PI 3,4-diphosphate and PI 3,4,5-triphosphate in response to stimulation of cells by platelet-derived growth factor. p85 was found to be partially coaligned with microtubules (Kapeller *et al.*, 1993). Mitogen-activated protein kinase (MAPK) associates with cytoplasmic, mitotic and cytokinetic microtubules (Reszka *et al.*, 1995). The results in those experiments suggested that a direct association of MAPK with MTs may be in part responsible for the observed correlations between MAPK enzymatic activities and cytoskeletal alteration. GTP-binding proteins, (G) proteins, are key molecules in signal transduction. Therefore, their interaction with tubulin is of particular interest. Participation of cytoskeletal elements in regulation of hormonal response and responsiveness has been suggested by several laboratories. Addition of tubulin dimers to rat cerebral cortex synaptic membranes causes stable inhibition of adenylyl cyclase, and the molecular basis for this effect appears to require a direct interaction between tubulin and G proteins, as has been demonstrated by Wang *et al.* (1990). This suggests that dimeric tubulin is involved in neuronal signal transduction, acting through Gs and Gi1 (Roychowdhury *et al.*, 1994), to activate adenylyl cyclase in rat cerebral cortex membranes (Hatta *et al.*, 1995). LIS1 is an unconventional MAP and does not contain any known MT binding motifs; it binds to both tubulin subunits and microtubules, as well as functions as a subunit of PAF-AH.

The newly identified function of LIS1 as an unconventional MAP is significant in the long term goal of understanding its function and involvement in causing the lissencephaly phenotype. We have previously shown that murine and human LIS1 are expressed in those regions of the brain undergoing neuronal migration (Reiner *et al.*, 1995), that are known to be affected in lissencephaly patients. *NudF* (Xiang *et al.*, 1995), which shares a homology to LIS1 (42% identity), is required for MT-

dependent nuclear migration in *Aspergillus nidulans*. Mutations in the *tubA α* tubulin gene can suppress the *nudF* mutation, as well as the dynein mutation, suggesting a genetic interaction between tubulin, dynein and nudF (Willins *et al.*, 1995). An additional homolog of LIS1 was identified in *Saccharomyces cerevisiae* (33% identity) (Geiser *et al.*, 1997) in a pathway that is involved in nuclear migration. Interestingly, yeast dynein mutants demonstrate an alteration in microtubule dynamics (Carminati and Stearns, 1997). Cell migration is characterized by dynamic interactions between the substrate and the cytoskeleton-associated motile apparatus inside the cell (Huttenlocher *et al.*, 1995). Furthermore, the role of MTs in the forming of growth cones and neuronal migration is well documented (Gordon, 1991; Avila, 1992). Thus the finding that LIS1 influences MT dynamics provides additional information about the molecular events governing neuronal migration that have so far not been completely elucidated (Rakic *et al.*, 1994). The unusual G-protein-like ($\alpha 1/\alpha 2$) β trimer structure of PAF-AH(Ib) suggests that LIS1 is an effector molecule that transmits the signals produced by intracellular PAF to the cytoskeleton. LIS1 may exert its influence by modulating MT dynamics during neuronal development. Future studies intended to elucidate the regulation of the interactions between the three PAF-acetylhydrolase subunits, the MT-cytoskeleton, LIS1 and possibly additional interacting proteins, will enable us to better understand this important facet of normal and lissencephalic brain development.

Materials and methods

Generation of anti-LIS1 antibodies

The complete open-reading frame of LIS1 (Reiner *et al.*, 1993) was amplified using PCR, cloned into the pRSET vector (Invitrogen), and the sequence was verified. The LIS1 protein (pRSET-LIS1) was purified using a nickel-agarose column according to the manufacturer's protocol (QIAgen, Hilden, Germany). Antisera to recombinant LIS1 were generated in rabbits and affinity purified according to conventional protocols (Harlow *et al.*, 1988). To obtain monoclonal antibodies, the described LIS1 recombinant protein was injected into mice. Spleens were fused to generate hybridomas. Hybridoma supernatants were screened using Western blots. Positive hybridomas were subcloned and used to generate ascitic fluid. The anti-LIS1 monoclonal clones used in the described experiments were clones 338.40 and 210.11.4.

Immunostaining procedure

Tissue culture cells were washed with PBS, permeabilized for 2 min with $1 \times$ M buffer (50 mM MES pH 6.5, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA) supplemented with 0.5% Triton X-100 and 4% PEG (mol. wt 8000), followed by three washes of $1 \times$ M buffer. Cells were fixed with 3% formaldehyde for 20 min, then washed three times with PBS. These cells were incubated with affinity purified, polyclonal anti-LIS1 antibodies or anti-tubulin (clone DM1A, Sigma), washed with PBS and then incubated with a fluorescent-labeled second antibody. Some cells were treated with nocodazole (5 μ M) overnight prior to permeabilization and fixation. Primary rat hippocampus neurons were received from Dr Futerman and were permeabilized and fixed as described (Schwarz *et al.*, 1996).

Purification of tubulin

Bovine brain tubulin obtained from freshly slaughtered animals was purified through two cycles of polymerization-depolymerization, followed by chromatography on a phosphocellulose column and a third cycle of polymerization-depolymerization, as described by Williams (1992), modified as described (Fygenson *et al.*, 1994; Fygenson, 1995).

Recombinant LIS1 expression in insect cells

The open reading frame of LIS1 was cloned into a baculovirus expression vector pAcSG His NT-A (Pharmingen, CA, USA) and transfected into

Sf9 cells using the BaculoGold[®] system, according to the manufacturer's protocol. Recombinant protein was extracted from the cells and purified on Ni-NTA beads (QIAgen, Hilden, Germany) under non-denaturing conditions.

MT binding assay (co-sedimentation)

P-11 phosphocellulose purified bovine brain tubulin (9 μ M) was incubated with 3 μ l of precentrifuged (96 000 g, 15 min at room temperature) *in vitro* translation mixture in 40 μ l PEM buffer (100 mM PIPES pH 6.9, 1 mM $MgSO_4$, 1 mM EGTA), 1 mM GTP, 1 mM ATP, taxol (20 μ M) or nocodazole (10 μ g/ml) were added as indicated in the text. The experiments designed to assay the affect of 0.4 M NaCl or anti-LIS1 antibodies were performed in the presence of taxol (20 μ M). The mixture was incubated for 30 min at 37°C and centrifuged to sediment polymerized microtubules at 39 000 g for 30 min at room temperature. The resulting supernatant fractions were collected and the pellets were rinsed with PEM buffer and then dissolved in electrophoresis loading buffer. Pellets and supernatants were analyzed by SDS-PAGE. The quantification was performed by phosphorimager (Fuji) analysis for radioactively labeled samples. The relative amounts of tubulin were measured by densitometry of Coomassie stained gels. In addition to the cosedimentation assays, we have performed similar experiments using pre-assembled taxol stabilized MTs with the translation products. No significant differences were observed.

Co-immunoprecipitation

In vitro translated protein (5 μ l) was added to the P-11 phosphocellulose purified bovine brain tubulin (5 μ M) in PEM buffer containing 1 mM ATP and 1 mM GTP solution and mixtures were incubated at room temperature for 20 min. The concentration of tubulin used in these experiments was lower than the critical concentration that allows spontaneous nucleation (Fygenson, 1995). After the incubation, mixtures were diluted to 500 μ l of PBS containing 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS (PBS-TDS), and the antibodies (~0.5 μ g) were added. This mixture was incubated for 30 min and then 20 μ l of agarose conjugate (protein A,G plus, Santa Cruz) were added and incubated for 2 h at 4°C. Immunoprecipitated proteins were pelleted by centrifugation (690 g, 3 min), washed three times with PBS-TDS, eluted by addition of SDS-PAGE sample buffer, boiled for 2 min and analyzed on SDS-PAGE.

BIAcore™ analysis

These experiments were performed on a BIAcore™ 2000 biosensor (Pharmacia Biosensor AB, Uppsala, Sweden). All experiments were performed at 25°C using a HEPES buffer pH 7.4 with 150 mM NaCl, 0.005% surfactant P-20 (Pharmacia), 1 mM $MgSO_4$, 1 mM EGTA, 1 mM GTP. The buffer flow rate was 10 μ l/min. Recombinant LIS1 was immobilized on a sensor chip and several surface densities were used as indicated using the amine coupling kit (Pharmacia), the protein was injected at 10 μ l/min in a coupling buffer at pH 6; the activation period was 6 min. Immobilized LIS1 was regenerated by injection of HBS with 1 M NaCl. The highest tubulin concentration used (1.8 μ M) is lower than the critical concentration allowing spontaneous nucleation at 25°C (Fygenson, 1995).

Microtubule dynamics analysis

The growing and shortening dynamics of individual microtubules were visualized by video-enhanced DIC microscopy. A solution (10 μ l) containing phosphocellulose-purified tubulin (10 μ M) in PEM buffer (0.1 M PIPES pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$) supplemented by GTP (1 mM), axonemes (one to three per field) and 1 μ l of LIS1 or LIS1 buffer (50 mM sodium phosphate buffer pH 8, 300 mM NaCl, 10% glycerol) were prepared. Preparation of axonemal fragments from sea urchin sperm was done according to Williams (1992). The samples were introduced into sealed cells and incubated for 10 min at 30°C before measurements were taken. DIC microscopy was carried out at 30°C, using a Zeiss microscope equipped with a temperature-controlled oil-immersion objective (Fluor 100/1.3) and a VCR. Images were captured in real time, using a CCD video camera (isight) connected to a monitor. MT images from the video or camera were frozen on the computer screen using Inspector™ (Matrox) software. Measurements of length were made at 30 s intervals (growing phase) or at 5 s intervals (shortening phase). An average of 20 MTs were used to calculate the results for each experiment. Length measurements were performed on plus ends until microtubules were depolymerized completely or until the image became obscure. Within this time frame, the highest value recorded was defined as maximal length. The catastrophe frequency was calculated

by dividing the number of catastrophes by the sum of total time spent in the growing phase by each fiber measured. The error in the transition frequencies was estimated by dividing the frequency by the square root of the number of transitions (Walker *et al.*, 1988).

Quantitative Western analysis

Determination of LIS1 and tubulin levels in bovine brain extracts was performed by quantitative immunoblotting. Bovine brain extracts were prepared as described previously. Recombinant purified LIS1 and purified tubulin were used as standards. Protein concentration was determined using Bradford reagent (Bio-Rad). For quantitative immunoblotting, bovine brain lysate (10, 25, 50 and 100 µg per lane; for each measurement each lane was loaded four times and averaged) and nine samples of purified protein (50 ng–1.4 µg tubulin, 50–250 ng LIS1) were loaded in triplicate on SDS gels. Immunoblots were probed with an anti-LIS1 monoclonal antibody (clone 210.11.4), polyclonal anti-LIS antibody or anti-α tubulin monoclonal antibody (DM1A, Sigma). Iodinated goat anti-mouse antibodies (10⁶ c.p.m./ml) or protein A (10⁶ c.p.m./ml) were used as secondary probes. Quantification was done by a phosphorimager after 2 h exposure.

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