Spastin interacts with the centrosomal protein NA14, and is enriched in the spindle pole, the midbody and the distal axon

Alessia Errico, Pamela Claudiani, Marilena D'Addio and Elena I. Rugarli*

Telethon Institute of Genetics and Medicine (TIGEM), via P. Castellino 111, 80131 Naples, Italy

Received May 25, 2004; Revised and Accepted July 12, 2004

Hereditary spastic paraplegia (HSP) is characterized by the specific retrograde degeneration of the longest axons in the central nervous system, the corticospinal tracts. The gene most frequently involved in autosomal dominant cases of this disease, SPG4, encodes spastin, an ATPase belonging to the AAA family. AAA proteins are thought to exert their function by the energy-dependent rearrangement of protein complexes. The composite function of these proteins is directed by their binding to regulatory factors and adaptor proteins that target their activity into specific pathways in vivo. We previously found that overexpressed spastin interacts dynamically with microtubules and displays microtubule-severing activity. Here, we demonstrate that spastin is enriched in cell regions containing dynamic microtubules. During cell division spastin is found in the spindle pole, the central spindle and the midbody, whereas in immortalized motoneurons it is enriched in the distal axon and the branching points. Furthermore, spastin interacts with the centrosomal protein NA14, and co-fractionates with γ-tubulin, a centrosomal marker. Deletion of the region required for binding to NA14 disrupts spastin interaction with microtubules, suggesting that NA14 may be an important adaptor to target spastin activity at the centrosome. These data strongly argue that spastin plays a role in cytoskeletal rearrangements and dynamics, and provide an attractive explanation for the degeneration of motor axons in HSP.

INTRODUCTION

Hereditary spastic paraplegia (HSP) is a genetically heterogeneous mendelian disorder characterized by weakness, spasticity and loss of the vibratory sense in the lower limbs (1–3). Some forms of HSP are complicated by additional neurological symptoms, but all are characterized by the retrograde degeneration of axons of the corticospinal tracts and the fasciculi gracilis (4,5). These tracts contain the longest axons in the central nervous system. HSP represents a model disease to study the processes underlying axonal degeneration, a pivotal cause of neurological impairment in several neurodegenerative diseases (6).

The most common form of autosomal dominant HSP, accounting for ~40% of the cases, is due to mutations in the SPG4 gene (7). Although it is classified as a pure form of HSP, some studies have identified a slight cognitive impairment as a clinical feature of patients with SPG4 mutations (8). Age of onset is variable, but usually the first symptoms arise in early adulthood. Nonsense, frame shift and missense mutations have all been found in SPG4 patients, and are characterized by an indistinguishable clinical phenotype, suggesting that the molecular basis of the disease is haploinsufficiency (9,10).

SPG4 encodes spastin, a 616 amino acid protein belonging to the broad AAA (triple A; ATPase associated with various cellular activities) family. The signature of the AAA family is a 240 amino acid conserved P-loop NTPase domain, which besides the Walker A and B motifs contains additional sequence conservation, the ‘second region of homology’. AAA proteins are involved in a wide variety of cellular processes, such as cell cycle, vesicular transport, mitochondrial function, peroxisome biogenesis and proteolysis (11–13). Spastin belongs to the poorly characterized subfamily 7, which also contains p60 katanin, a microtubule-severing protein (14), and SKD1, a regulator of endosome trafficking (15). Interestingly, the vast majority of missense mutations identified in HSP patients are located in the AAA domain.

AAA proteins are viewed as molecular chaperones that couple ATP hydrolysis to the disassembly and unfolding of...
protein complexes. The manifold cellular functions of AAA proteins are conferred by the combination of their subcellular localization and the interaction with adaptor proteins that bring them in contact with specific substrates (16). In some cases, the binding of a single AAA protein to multiple molecules is reflected in its action in seemingly unrelated processes (17,18). All known AAA proteins have a conserved structure, with one of two AAA domains located in the C-terminal part of the protein, whereas the N-terminal part contains recognition sites for substrates and adaptors. Spastin N-terminal region is still poorly characterized, but contains the MIT (found in microtubule-interacting and trafficking molecules) domain, a recently identified protein motif, which is common to proteins involved in endocytosis and trafficking, such as SKD1 and SNX5 (19). Notably, the MIT domain is also present in spartin, the product of the SPG20 gene, mutations in which cause Troyer syndrome, an autosomal recessive form of HSP complicated by mental retardation (20).

The function of spastin is largely unknown. Previous studies performed in our laboratory suggested a role for spastin in microtubule dynamics (21). We found that overexpressed spastin interacts transiently with microtubules through its N-terminal region, and that this interaction is regulated through the ATPase activity of the AAA domain. Furthermore, we showed that the microtubule network in cells overexpressing spastin is disrupted. We therefore hypothesized that spastin might be implicated in microtubule severing, as shown for the highly homologous p60 katanin, and that impairment of fine regulation of the microtubule cytoskeleton in long axons may underlie pathogenesis of hereditary spastic paraplegia. However, there is still no evidence supporting a role for spastin in cytoskeletal dynamics in vivo.

One major caveat is subcellular localization of spastin. Two recent studies have reported partially discrepant results. Charvin et al. (22) found spastin in the nuclei of neuronal cells, whereas a more recent study detected it in the cytoplasm and/or in the nucleus depending on the neuronal population (23). Spastin was observed in the cytoplasm and synaptic terminals of cortical and hippocampal pyramidal cells of the cerebral cortex and hippocampus, of Purkinje cells and of spinal motoneurons (23). This composite picture possibly underlies a multifaceted cellular role of spastin.

As a strategy to identify the molecular pathways in which spastin is involved, we combined studies of the subcellular localization of endogenous spastin and searches of spastin adaptor proteins by yeast two-hybrid screenings. Here, we present findings indicating that spastin is enriched in cell regions containing dynamic microtubules, such as the centrosomes and the midbody in dividing cells, and the distal axons in motoneurons. As further evidence for a role of spastin in the cytoskeleton, we demonstrate that spastin interacts with the centrosomal protein NA14. The region of spastin interaction with NA14 overlaps with the minimal domain required for microtubule binding in vitro, suggesting that NA14 may be an important adaptor to direct spastin to exert its activity on microtubules at the centrosome. These data strongly argue that one of the pathways in which spastin is implicated may be cytoskeletal dynamics and rearrangement in both proliferating and postmitotic cells.

RESULTS
Spastin localizes to the spindle poles and the midbody during cell division

To get more insights on spastin subcellular localization, we developed rabbit polyclonal antibodies against different regions of spastin and purified them by affinity chromatography. Two affinity-purified antibodies R74 and S51 (directed against spastin fusion proteins comprising amino acids 1–122 and 87–354, respectively) specifically identify transfected spastin as a 68 kDa protein (Fig. 1A and B). Both antibodies immunoprecipitate an endogenous protein of the same size of transfected spastin from HeLa cell lysates (Fig. 1A and B, black arrowhead). Furthermore, this protein is detected using the S51 antibody for immunoprecipitation and the R74 for western blot (Fig. 1C), and vice versa (data not shown). This protein is not immunoprecipitated by the preimmune serum and disappears after competitive preincubation with the antigens (data not shown). We therefore conclude that the 68 kDa immunoprecipitated protein corresponds to endogenous spastin. We do not detect spastin in total cell lysates by western blot unless we load an extremely high amount of proteins, indicating that spastin is a low-abundant protein. Affinity-purified S51 also identifies an additional protein of about 62 kDa in total cell lysates (Fig. 1A, gray arrowhead). We do not know whether this 62 kDa protein represents a cross-reacting band or a spastin isoform. This band is specifically competed by preincubation with the antigen, but is not enriched by spastin transfection.

As the R74 antibody clearly detects only spastin by immunoprecipitation and western blot (Fig. 1B and C), we used it to perform immunofluorescence experiments on different cell types. Spastin is detected as a diffuse nuclear signal in HeLa and Cos7 cells. This staining is not identified by the preimmune sera, and is competed specifically by preincubation with the antigen (Fig. 2A–C). These data are consistent with what has been previously described (22).

We then analyzed spastin subcellular localization in cells undergoing division. During metaphase, spastin signal concentrates in the centrosomes and on spindle microtubules (Fig. 2D). This signal is still present on the spindle poles during anaphase and is successfully competed by preincubation with the antigen (Fig. 2E and F). Spastin is also enriched at the midbody during cytokinesis (Fig. 2G). To confirm this data, we repeated these experiments with the S51 affinity-purified antiserum. This antibody stains only very weakly the nucleus of cells and the spindle poles (Fig. 2H and I, and data not shown), but decorates the central spindle during anaphase (Fig. 2H and I, arrowhead) and the midbody (Fig. 2H and K–M, arrows). These signals are competed by preincubation of the antibody with the antigen (Fig. 2O). The central spindle or midzone forms in late anaphase between the two separating sets of chromatids and consists of a dense network of overlapping antiparallel microtubules. As a cleavage furrow forms and ingresses, the microtubules of the central spindle are pushed in a bridge that tethers the two daughter cells, the midbody. Both the central spindle and the midbody are required for progression of cytokinesis. It is noteworthy that spastin is always located at the central region of the midzone and of the midbody,
as shown by co-localization with α-tubulin (Fig. 2J). At the late stage of cytokinesis, when microtubules are pulled aside, spastin is located at the center of the intercellular bridge (Fig. 2K–M).

These data indicate that spastin undergoes dynamic changes in its subcellular localization during the cell cycle. Spastin is mainly nuclear in interphase cells, and becomes associated with the centrosomes, the spindle microtubules, the midzone and finally the midbody during cell division. All these regions are characterized by striking changes in the dynamic properties of microtubules.

**Spastin localizes to distal regions of axons**

We then sought to determine spastin subcellular localization in postmitotic neurons, and we employed the NSC34 cell line. NSC34 is a hybrid cell line obtained by fusing the aminopterin-sensitive neuroblastoma N18TG2 with motor neuron-enriched embryonic day 12–14 spinal cord cells. When NSC34 cells are plated on a matrigel substrate and allowed to differentiate, they acquire a multipolar neuron-like phenotype and possess most of the motoneuronal properties (24). Consistent with the findings in HeLa, the R74 antibody stains the midbody in dividing NSC34 cells (Fig. 2P and Q).

In differentiated postmitotic NSC34, the R74 antibody labels discrete nuclear domains in agreement with previous findings (22), but most interestingly detects a specific signal in the neurites (Fig. 3A). This signal is not uniformly distributed, but is characteristically enriched in the distal axon and in the branching regions (Fig. 3A and D). Spastin is excluded from the most peripheral region of the growth cone, which has highest F-actin content (compare Fig. 3B and C). This cytoplasmic staining of neurites is highly reminiscent of that observed for tau and MAP1b, two microtubule-associated proteins that are downregulated in axons of adult motoneurons where they are only detected in their distal region (25, 26). To test this possibility, we double-stained motoneuronal cultures with the R74 antibody and the RT97 monoclonal antibody, which recognizes phosphorylated epitopes of high molecular weight neurofilaments and the microtubule-associate proteins tau and MAP1b (27). Co-localization of the two signals was observed by the confocal microscopy (Fig. 3E and F).

**Isolation of NA14, a centrosomal protein, as a spastin interactor**

The complex subcellular localization of endogenous spastin strongly indicates that the protein may serve very different roles, depending on the cell type and the cell cycle. A composite function is not unusual for AAA proteins, which are frequently involved in different pathways, depending on their localization and binding to adaptor proteins. The identification of molecular partners therefore represents a crucial step in identifying these functional pathways.

To isolate candidate spastin interacting molecules, we performed a yeast two-hybrid screening of a human fetal brain cDNA library, using full-length human spastin as bait. Screening of 0.5 × 10^6 independent clones led to the isolation of 30 identical clones, corresponding to a partial CDNA, encoding the protein NA14. To ensure that this interaction is specific, the prey was reintroduced into yeast and its ability to grow in the absence of leucine and induce β-gal activity with the original spastin bait was confirmed (Fig. 4).

NA14 is a 119 amino acid protein that was originally identified as one of the autoantigens recognized by the serum of a patient with Sjogren syndrome (28), and later found to be a component of mammalian centrosomes (29,30). The localization of NA14, combined with our finding that spastin associates with centrosomes during cell division, prompted us to investigate this interaction in greater detail.

The NA14 clone retrieved by the two-hybrid screening lacks the first 33 amino acids, but comprises an almost complete coiled-coil region and the entire C-terminal basic domain of the protein (28). To identify the region of spastin that is involved in the interaction with NA14, we determined whether spastin deletion constructs would still bind NA14 in the two-hybrid system. This analysis showed that the region of spastin responsible for the interaction with NA14 is located between amino acid 50 and 87 in the N-terminal region (Fig. 4). During these studies we also found evidence that NA14 self-associates, as previously reported (28,29) (Fig. 4).

**Spastin and NA14 interact in vivo**

To determine whether the interaction between spastin and NA14 also occurs in vivo, we performed co-immunoprecipitation
experiments in mammalian cells. NA14 is expressed at low levels in several mammalian cell lines, including HeLa, hampering the immunoprecipitation of the endogenous protein from these cells (28). We therefore cloned the full-length NA14 cDNA in frame with the HA epitope in the pCDNA3 vector, and tested whether endogenous spastin and overexpressed HA–NA14 could be co-immunoprecipitated. This experiment showed that NA14 was detected in the HeLa cell lysate immunoprecipitated by the S51 antibody but not by the preimmune serum (Fig. 5A). The S51 antibody detected a weak band corresponding to endogenous spastin, after immunoprecipitation with the anti-HA antibody (data not shown). These data suggest that NA14 and spastin can interact in vivo.

Figure 2. Subcellular localization of endogenous spastin in proliferating cells. Immunofluorescence analysis was performed using affinity-purified anti-spastin antibodies R74 and S51. HeLa cells stained with the R74 antibody show a prevalent spastin nuclear signal (A), which is not present using the preimmune serum (B) and is competed by the antigen (C). In Cos7 cells undergoing cell cycle, the R74 antibody detects a specific signal on the spindle poles during metaphase (D) and late anaphase (E) and at the midbody (G, arrows). These signals are efficiently competed by the antigen (compare E and F). The S51 antibody reveals a weaker signal on HeLa cell nuclei (H). During cell division, spastin signal is enriched in the midbody (H, K–M, arrows) and in the central spindle (H, I, arrowhead). In these regions, spastin localizes to microtubules as assessed by double staining with the anti-α-tubulin antibody (J). In the midbody, the S51 antibody detects a signal at the center of the intercellular bridge when the cell is in an advanced cytokinesis (K–M, insets). All these signals are not present in the preimmune serum staining (N) and are competed by the antigen (O). In dividing NSC34 cells, the R74 antibody stains the midbody (P) as assessed by double staining with the α-tubulin (Q).
Spastin is enriched in purified centrosomal fractions

To gain further evidence for the association of spastin to the centrosome, we asked whether spastin is enriched in centrosomal fractions. We purified centrosomes from HeLa cells by a discontinuous sucrose gradient ultracentrifugation, after removal of swollen nuclei, chromatin aggregates and unlysed cells. This method allows the concentration of centrosomes from an extremely high number of cells (31). We used the S51 antibody to detect spastin in the different cytoplasmic fractions, and anti γ- or β-tubulin antibodies to distinguish the centrosome-containing fractions. Spastin was detected in the fractions containing γ-tubulin, and not in those containing β-tubulin as expected for a centrosomal protein (Fig. 5B). The same result was obtained using the R74 antibody (data not shown).

The NA14 binding region is involved in microtubule interaction

We have provided evidence that a fraction of spastin is enriched in the centrosome and in microtubule structures during different phases of the cell cycle in proliferating cells. Moreover, spastin levels are increased in the distal axon, a region where microtubules are highly dynamic. We previously showed that spastin contains a region of interaction with microtubules within its N-terminus (amino acids 1–263) (21). Overexpressed spastin co-sediments with microtubules (21). The spastin–microtubule interaction is regulated by the activity of the AAA domain, and can be best revealed by expressing spastin mutants unable to bind and hydrolyze ATP. In fact, when these mutants are expressed in Cos7 cells, they bind stably to a subset of microtubules (21). As we mapped the region implicated in binding to NA14 within the N-terminal region of spastin, we asked whether binding to NA14 contributes to the microtubule localization of spastin.

To this end, we refined the mapping of the spastin microtubule-interacting region by transfecting a series of spastin deletion constructs, devoid of different parts of the N-terminal region, and testing their ability to co-sediment with taxol-stabilized microtubules. Deletion of the first 50 amino acids of spastin still allows co-sedimentation with polymerized microtubules, whereas removal of the first 87 amino acids prevents it, indicating that the region of spastin between amino acids 50 and 87 is crucial for interaction with microtubules (Fig. 6A). To corroborate our findings, we analyzed by immunofluorescence a similar series of N-terminal-deleted spastin constructs bearing the K388R
mutation and therefore unable to bind ATP (Fig. 6B–F). Again, we observed that deletion of the region between amino acids 50 and 87 was sufficient to drastically modify the subcellular localization of the transfected protein, which appeared more diffuse and almost completely lost the ability to co-localize with tubulin (Fig. 6D). These data suggest that an important region of interaction with microtubules is located between amino acids 50 and 87 and overlaps with that required for binding to NA14. It is interesting to point out that deletion of the MIT domain does not affect spastin interaction with the microtubule network (Fig. 6A and F).

DISCUSSION

ATPases of the AAA family couple their enzymatic activity to the remodeling of protein complexes, and are involved in different cellular activities on the basis of their subcellular localization and their binding to specific adaptors (12,16). We previously showed that spastin acts as a microtubule-severing protein when overexpressed in mammalian cells, whereas expression of spastin mutants unable to hydrolyze ATP brings to the formation of stable bundles of microtubules, suggesting that spastin is involved in microtubule dynamics (21). Here, we present evidence that spastin has a complex subcellular localization, dependent on the cell type and the cell cycle, pointing to a role of spastin in different pathways. Many of the sites of spastin association are characterized by dynamic changes of the microtubule network, suggesting that these are the regions where spastin exerts its microtubule-severing activity.

We demonstrate that spastin is highly enriched in cellular centrosomal fractions. Moreover, anti-spastin antibodies decorate the spindle poles during cell division, indicating that spastin may concentrate at the centrosome in a cell cycle dependent manner, as demonstrated for many cellular effectors. The centrosome is a tiny, non-membranous organelle usually in close proximity to the nucleus, composed of centrodes and pericentriolar material. This is a dynamic matrix of fibers and protein aggregates, and provides a scaffold for anchoring proteins that are involved in microtubule nucleation and other activities (32). Spastin may be implicated in severing the microtubule network during mitosis, similarly to p60 katanin (33).

p60 katanin forms an heterodimer with p80 katanin, a WD-40 containing protein, which targets the p60 subunit to the spindle poles and positively regulates its microtubule-severing activity (33,34). In a search for spastin interacting molecules, we have identified the centrosomal protein NA14 and showed its ability to co-immunoprecipitate with endogenous spastin in vivo. NA14 was originally isolated as one of the two major autoantigens recognized by the serum of a patient with Sjögren syndrome, an autoimmune disease characterized by dry eyes and mouth (28). The first hint of a possible localization of NA14 to the centrosome came from a study on its Chlamydomonas reinhardtii ortholog, DIP13 (29). Chlamydomonas is a unicellular bilaflagellate green alga that possesses a well-defined microtubule cytoskeleton. DIP13 was found to localize to cytoplasmic microtubules, as well as to basal bodies and flagellar axonemes, which are very similar in structure and function to mammalian centrioles and cilia, respectively. Interestingly, the anti-DIP13 antibody also recognized human NA14 by immunofluorescence and stained basal bodies and flagella of human sperm cells, as well as the centrosome of HeLa cells (29). With a completely independent approach, NA14 was later confirmed to be a component of human centrosomes in a mass spectrometry-based proteomic analysis (30).

The role of NA14/DIP13 at the centrosome is completely unknown. Antisense experiments, in which the levels of DIP13 in Chlamydomonas were reduced, resulted in the appearance of multinucleate and multiflagellate cells,
suggested that DIP13 may be implicated in cell division (29). The authors conceive that DIP13/NA14 may represent a conserved protein family involved either in a general stabilization of microtubules or in linking microtubular structures to motile systems. The presence in NA14 of a coiled-coil domain makes it a perfect candidate to be a structural centrosomal component. We propose that NA14 is a molecular adaptor involved in targeting spastin to the centrosome, thus triggering the spastin microtubule-severing activity in a specific cellular location and with an exact timing. In support to the hypothesis that NA14 represents a molecular anchor for spastin, we found that deleting the region of spastin involved in the interaction with NA14 abrogates the ability of an ATPase defective mutant to associate with microtubule bundles in vitro, and abolishes co-sedimentation of wild-type spastin with microtubules.

The centrosome is not the only site where spastin contacts the microtubule cytoskeleton. In fact we found that spastin is also highly enriched in the midzone or central spindle and at the midbody during cytokinesis. Cytokinesis is a highly regulated process by which two daughter cells physically separate after cell division (35,36). A variety of molecules essential for cytokinesis have been localized at the central spindle and at the midbody. Spastin is enriched in these two structures, initially co-localizing with the most central subset of microtubules, and later being restricted to the innermost dense region of the midbody where abscission occurs. Remarkably, the mother centrosome migrates into the midbody to trigger the final steps of cytokinesis, and the attractive hypothesis has been put forward that the centrosome would deliver to the midbody a specific signal required for abscission, thus serving as a checkpoint for cytokinesis (37). Spastin may be important to trigger disassembly of the central spindle microtubules and midbody abscission, through its microtubule severing activity. Binding to regulatory proteins other than NA14 may be required for targeting spastin to the central spindle and midbody in vivo. Alternatively, the N-terminus of spastin may be able per se to interact with the cytoskeleton. In fact, a rearrangement of the microtubule cytoskeleton is observed in cells transfected with all types of spastinK388R mutants (Fig. 6), whereas all N-terminally deleted wild-type constructs induce microtubule-severing (data not shown). Association of transfected spastin with the endogenous protein could also underlie these results.

Our data suggest that spastin plays a role in cell division and cytokinesis, and identify NA14 as one of the partners involved. These processes are not affected in HSP patients, who carry SPG4 mutations in the heterozygous state. However, this result is not completely at odds with the neurological phenotype of HSP patients. Indeed, it has been proposed that proliferating cells and terminally postmitotic neurons use the same mechanisms and same group of molecular actors to organize their microtubules (38), and there are multiple examples of proteins with dual function (38–40). Among these, p60 katanin is implicated in releasing microtubules from the centrosome during mitosis, as well as in the mechanisms that regulate microtubule length in axons of postmitotic neurons (33,41).

We find that spastin is present in the cytoplasm of axons of immortalized motoneurons in vitro, as also observed in human brain specimens (23). Notably, spastin signal is not uniform, but undergoes a progressive proximal to distal increase, being enriched in the growth cone with the exclusion of its most peripheral region, and in branching points. The distal growth cone and the branching regions are sites of high dynamic events involving microtubules and F-actin (42,43). In fact, growth, extension and precise steering of axons toward synaptic targets depend on a tight control of the dynamics of the cytoskeletal components. To accomplish these tasks, neurons possess a variety of isoforms and of posttranslational modifications of cytoskeletal proteins and make use of a vast set of microtubule-associated proteins (44). Stathmin, a microtubule destabilizing protein, is enriched in the central domain of the growth cones (45). Mice knockout for stathmin display a late-onset axonal degeneration phenotype remarkably similar to HSP, suggesting that these mechanisms are also important for axon integrity in adult life (46). We speculate that spastin influences microtubule dynamics in growth cones, thus regulating the stability of axons and
axonal transport. These processes are expected to be complex and redundant, but long motor axons of the corticospinal tract could be highly dependent on spastin function. Axonal degeneration may occur as a result of inefficient severing of microtubules when spastin levels are reduced to half dosage, owing to haploinsufficiency. However, we cannot rule out the hypothesis that the pathogenic mechanism of missense mutations may be dominant-negative, and depend in part on the interference of mutant spastin with the molecular motors that transport cargos on microtubules, as suggested by a study in which mutant spastin was overexpressed in neurons (47).

In conclusion, our findings suggest that spastin is a multifaceted protein, with a complex function dependent on the cell type, the cell cycle and the molecular interactor. A survey of spastin involvement in dynamic remodeling of the microtubule cytoskeleton in developing and adult axons promises to be an appealing avenue to follow in order to understand the pathogenesis of HSP. However, this may be only one of the pathways in which spastin is implicated; we and others (22,23) also found spastin in the nucleus of neurons and the exploration of the nuclear function of spastin could reveal other interesting roles of this versatile AAA molecule.

MATERIALS AND METHODS

Generation of spastin specific antibodies

Two different constructs were used for the production of specific rabbit polyclonal anti-spastin antibodies. A 6His–spastin fusion protein was made by cloning the portion of spastin between amino acids 1 and 122 into the BamHI and HindIII sites of the pQE30 vector (Qiagen). This fusion protein was expressed in bacteria and purified under denaturing condition according to the manufacturer’s instructions (Qiagen). A specific anti-spastin rabbit polyclonal antiserum, R74, was produced at PRIMM (Milan, Italy) using this antigen. A different portion of spastin, corresponding to amino acids 87–354, was obtained by PCR amplification and subcloned into the BamHI and EcoRI sites of pGEX-3X vector (Amersham Pharmacia) to generate a GST–spastin fusion protein. The GST–spastin fusion protein was expressed in bacteria, purified under native condition as described elsewhere (48) and injected in rabbits at Biogenes (Germany).

The anti-spastin rabbit antiserum, S51, was obtained. Both R74 and S51 antisera were affinity purified against their antigens with the NHS Hi-Trap™ affinity columns (Amersham Pharmacia), according to the instructions provided by the manufacturer.

Constructs for expression in mammalian cells

N-terminal deleted spastin cDNAs (Δ50-spastin, Δ87-spastin and Δ190-spastin) were amplified by PCR using appropriate primers and subcloned into the pMT21-myc vector (EcoRI/SalI). An ATG was recreated at the beginning of each construct. The ΔMIT spastin construct is deleted of the region between nucleotides 340 and 600. This construct was prepared by substituting the fragment EcoRI–XbaI (nucleotides 1–600 of spastin open reading frame) of the pMT21-spastin-myc clone (21) with a shorter region corresponding to nucleotides 1–340, previously amplified by PCR.

To introduce the point mutations K388R, in vitro mutagenesis was performed using the Quickchange site directed mutagenesis kit (Stratagene, La Jolla, CA, USA), as previously described (21).

Full-length NA14 cDNA was amplified by RT–PCR using HeLa cDNA as template and specific primers designed on available sequence (28). The resulting fragment was subcloned into the EcoRI and XhoI sites of the pcDNA3-HA vector in frame with the HA tag at its N-terminus. In each case the integrity of the clones was determined by direct sequencing.

Cell culture, cell transfection and immunofluorescence

Monkey kidney Cos-7 and HeLa cells were maintained in exponential growth in Dulbecco’s modified Eagle’s medium (DMEM, Highclone) containing 10% fetal bovine serum (Highclone). Immortalized motoneuronal cells NSC34 were kindly provided by Dr Neil R. Cashman (McGill University, Montreal, Canada) and were cultured in DMEM supplemented with 5% defined fetal bovine serum (Highclone). All constructs were transfected using Polyfect (Qiagen) according to instructions provided by the manufacturer. For immunofluorescence experiments, cells were grown on coverslips or on multiwell chamberslides (Nunc) and fixed for 5 min with a solution of 4% paraformaldehyde in PBS (S51) or in methanol at −20°C (R74). For NSC34 immunofluorescence, cells were plated on coverslips precoated with Matrigel (BD) (1:50 in DMEM) and fixed in methanol. Cells were permeabilized in PBS/0.2% Triton X-100 for 10 min, and blocked in 10% pig serum/PBS for 30 min. Cells were then incubated

Figure 6. Dissection of the spastin microtubule interacting region. (A) Cos7 cells were transfected with different N-terminal-deleted spastin constructs. Lysates from transfected cells were either supplemented or not with taxol. After sedimentation on sucrose cushion, supernatant (S) and pellet (P) fractions were analysed for the presence of spastin and tubulin, using the S51 and β-tubulin antibodies, respectively. When taxol is added to the lysates to stabilize polymerized microtubules, both Δ50- and ΔMIT-spastin proteins are detected in the pellet together with tubulin, whereas Δ87-spastin, and Δ190-spastin remain in the supernatants. As control, when lysates are not treated with taxol, all the proteins are detected only in the supernatant fractions. (B–F) Constructs shown at the right of each panel were transfected in Cos7 cells and double immunofluorescence experiments were performed to reveal spastin and tubulin localization. The blue box represents the domain of interaction with NA14 (amino acids 50–87), the green box indicates the MIT domain (amino acids 116–194) and the red box represents the AAA domain. In B, spastin is tagged with GFP (green signal), whereas tubulin is red. In C–F, spastin is revealed with the S51 antibody (red signal), whereas tubulin is stained with a monoclonal α-tubulin antibody (green signal). Nuclei are stained with DAPI (blue signal). All constructs carry the K388R mutation that impairs ATP hydrolysis and leads to constitutive binding of the full-length spastin to microtubule bundles (B). Deletion of the first 50 amino acids of spastin (C) or of the MIT domain (D) does not change this pattern, whereas removal of the first 190 amino acids shows an almost completely diffuse spastin signal (E). The same result is obtained by expressing a spastin deletion mutant devoid of the first 87 amino acids, indicating that the region between amino acids 50 and 87 is important for binding stably to microtubules in this assay (D). Note a rearrangement of the microtubule cytoskeleton in all cases.
with primary antibodies for 2 h at room temperature and appropriate secondary antibodies for 1 h. To detect nuclei, cells were stained using DNA specific stain DAPI (1 μg/ml, Roche). Cells were mounted with Vectashield (DBA) and examined with an Axiocam microscope (Zeiss) equipped with an Axiocam CCD camera and Axiovision digital imaging software (Zeiss). Alternatively, cells were analyzed with a confocal microscope (Leica).

For immunofluorescence the affinity purified spastin specific antibodies were used 1:5. Monoclonal anti-α-tubulin (1:250) was from Molecular Probes. Monoclonal RT97 was from Development Studies Hybridoma Bank (University of Iowa, IA, USA) (49) (1:50). All the secondary antibodies for immunofluorescence were from Dako (1:200).

**Yeast two-hybrid screening**

The two-hybrid screening was performed as previously described (50,51). As bait we used full-length spastin cloned in the pEG202 vector fused to the LexA DNA-binding domain. Two reporter genes were used in the screen, the LexAop–LEU2 gene that allows growth in absence of leucine and the LexAop–LacZ gene that directs the synthesis of the β-galactosidase (50). The bait was transformed into the yeast strain EGY48 that was subsequently transformed with a human fetal brain cDNA library cloned into pJG4-5, containing the B42 activation domain (kindly provided by Gianni Del Sal, Trieste). Transformants (0.5 × 10⁵ independent clones) were seeded on plates containing either X-gal or lacking leucine to select positive clones that have activated both LexA-driven reporter genes (lacZ and LEU2). Interaction mating assay was performed using the same system and two different mating types (EGY48 MAT α and EGY42 MAT a) as described (52). N-terminal deleted spastin cDNAs were amplified by PCR using specific primers and subcloned in the yeast pEG202 (BamHI/NorI) vector in frame with LexA DNA-binding domain. The pEG202-spastin construct, lacking the first 243 amino acids of spastin, was generated by digesting the pEG202–spastin construct with BamHI–NcoI (Biolabs), followed by KlenowI (Biolabs) treatment at room temperature for 15 min and self-ligation. The partial NA14 cDNA isolated from the human fetal brain library was subcloned by restriction digestion (EcoRI/XhoI) in the pEG202 vector fused to the LexA DNA binding domain.

**Immunoprecipitation and western blotting**

To immunoprecipitate endogenous spastin, 1 × 10⁷ cells were lysed in PEM/DNNA (Pipes 80 mM pH 6.8, EGTA 1 mM, MgCl₂ 1 mM, DTT 1 mM, NaCl 150 mM, NP-40 1%), supplemented with 1 mM protease inhibitor cocktail (Sigma). Samples were subjected to brief sonication (3 × 15 s) and then centrifuged at 10 000 g for 5 min at 4°C to remove cellular debris. Total extracts were precleared with protein A sepharose beads (Sigma) for 1 h at 4°C. The precleared lysates were supplemented with the appropriate primary antibody, incubated at 4°C for 3 h and the immunocomplexes were then collected on protein A sepharose beads (Sigma). Immunoprecipitateds were washed with PBS/0.1% Triton X-100 at least four times. Immunocomplexes and protein samples were resuspended in SDS sample buffer (20 mM Tris–HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 2.5% glycerol and 2.5% bromophenol blue) and subjected to standard SDS–PAGE electrophoresis followed by transfer to a polyvinylidene difluoride membrane (PVDF, Amersham). Western blot analysis was conducted with the enhanced chemiluminescence (ECL) reagents according to the manufacturer’s protocol (Amersham). For co-immunoprecipitation with NA14, 48 h postransfection of the pcDNA3–HA–NA14 construct, cells were collected and lysed in PEM/DNNA buffer and immunoprecipitation was carried out as described earlier, using the appropriate primary antibodies. Affinity-purified R74 was used 1:100 for immunoprecipitation (IP) and 1:1000 for immunoblot (WB); S51 1:100 (IP) and 1:2000 (WB). Monoclonal anti-HA antibody was used for immunoprecipitation (Sigma, 1:100), whereas polyclonal anti-HA for western blot detection (Sigma, 1:500). Horseradish peroxidase (HRP)-conjugated antibodies were from Amersham Pharmacia (1:3000).

**Centrosome purification**

Centrosomes were isolated from HeLa cells by discontinuous gradient ultracentrifugation according to the method of Moudjou and Bornens (31). Cells (1 × 10⁸) in the exponential phase of growth were treated with 1 μg/ml cytochalasin D and 0.2 μM nocodazole for 1 h at 37°C. Cells were trypsinized and collected by centrifugation. The resulting pellet was washed in TBS (10 mM Tris–HCl pH 7.4, 150 mM NaCl) and successively in 1 × TBS/8% sucrose. Cells were resuspended in 2 ml of 0.1 × TBS/8% sucrose, followed by the addition of 8 ml of lysis buffer (1 mM HEPES pH 7.2, 0.5% NP-40, 0.5 mM MgCl₂, 0.1% β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotenin and 1 mM PMSF) to obtain a final concentration of 1 × 10⁷ cells/ml. The suspension was gently shaken and pipetted several times through a 10 ml serological pipette. The lysates were spun at 2500 g for 10 min to remove the swollen nuclei, chromatin aggregates and unlysed cells. The resulting supernatant was supplemented with HEPES buffer to a final concentration of 10 mM and 1 μg/ml DNaseI, and incubated on ice for 30 min. The mixture was gently underlaid with 1 ml of 60% sucrose solution (10 mM Pipes pH 7.2, 0.1% Triton X-100 and 0.1% β-mercaptoethanol containing 60% sucrose w/v) and spun at 10 000 g for 30 min to sediment centrosomes on the cushion. The upper 8 ml of the supernatant was removed and the remaining part, containing the concentrated centrosomes, was vortexed and loaded onto a discontinuous sucrose gradient consisting of 70, 50 and 40% w/v solution from the bottom (500 μl 70% sucrose, 300 μl 50% sucrose and 300 μl 40% sucrose), and spun at 120 000 g for 1 h. An amount of 200 μl fractions were collected and centrosomes of each fraction were sedimented by addition of 1 ml of 10 mM Pipes pH 7.2 and centrifugation at 20 000 g for 15 min at 4°C. Supernatants were removed and centrosomes were resuspended in SDS sample buffer, fractionated on SDS–PAGE and analysed by western blotting. Monoclonal anti-β-tubulin (1:1000), and polyclonal anti-γ-tubulin (1:1000) were from Sigma.
Microtubule co-sedimentation

The microtubule cosedimentation experiments were carried out as previously described (21).

ACKNOWLEDGEMENTS

We wish to thank Gennaro Andolfi for technical assistance, Alex Reyon and Gianni del Sal for helpful suggestions and reagents for the two-hybrid screening, Neil R. Cashman for providing the NSC34 cell line, Rosa M. Rios for sharing with us unpublished data, Michel Bornens for suggestions with the centrosome purification and Germana Meroni and Graciana Diez-Roux for helpful discussion. This work was supported by the Italian Telethon Foundation, the European Union (contract number LSHM-CT-2003-503382) and the Italian Ministry of Health.

REFERENCES


