The Drosophila Gene abnormal spindle Encodes a Novel Microtubule-associated Protein That Associates with the Polar Regions of the Mitotic Spindle

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Abstract. abnormal spindle, a gene required for normal spindle structure and function in Drosophila melanogaster, lies immediately adjacent the gene tolloid at 96A/B. It encodes a 220-kD polypeptide with a predicted pI of 10.8. The recessive mutant allele asp<sup>1</sup> directs the synthesis of a COOH terminally truncated or internally deleted peptide of ~124 kD. Wild-type Asp protein copurifies with microtubules and is not released by salt concentrations known to dissociate most other microtubule-associated proteins. The bacterially expressed NH<sub>2</sub>-terminal 512-amino acid peptide, which has a number of potential phosphorylation sites for p34<sup>cdc2</sup> and MAP kinases, strongly binds to microtubules. The central 579-amino acid segment of the molecule contains one short motif homologous to sequences in many of actin bundling proteins and a second motif present at the calmodulin binding sites of several proteins. Immunofluorescence studies show that the wild-type Asp protein is localized to the polar regions of the spindle immediately surrounding the centrosome. These findings are discussed in relation to the known spindle abnormalities in asp mutants.

Mutations in abnormal spindle (asp)<sup>1</sup> lead to defects in mitosis at a variety of developmental stages as well as in male meiosis. The late larval–pupal lethality seen with strong hypomorphic alleles of asp is typical of mutations in Drosophila genes encoding stable proteins essential for mitosis. Homozygous individuals receive sufficient maternal gene product from their heterozygous mothers to permit normal embryonic development to proceed. However, there is insufficient functional gene product to permit mitosis during larval and pupal stages, when the imaginal discs and abdominal histoblast nests proliferate to form adult structures. Rare escapers may display a number of cuticular abnormalities associated with cell death, such as missing bristles, misshapen tergites and sternites, and roughened eyes. The maternal contribution of Asp protein has been demonstrated directly in cuticular clones in which there has been the loss of a marked Y chromosome as a result of nondisjunction due to the mutation (Carmena et al., 1991). The frequency and size of such clones correlate inversely with the amount of maternally contributed asp<sup>1</sup> gene product.

The asp locus was originally identified through a late larval lethal mutation that causes defects in both mitosis and meiosis (Ripoll et al., 1985). Several mitotic abnormalities may be observed in third instar larval neuroblasts from asp homozygotes. There is an increased mitotic index and a high frequency of polyploid cells. Many metaphase figures have very highly condensed chromosomes, suggestive of a delay in progress through metaphase, and anaphase figures appear abnormally broad. asp<sup>1</sup> animals show reduced fertility, and a high frequency of nondisjunction has been observed in both divisions of male meiosis. Phase contrast microscopic analysis of meiosis in homozygous asp males revealed the abnormalities of spindle structure that gave the locus its name (Ripoll et al., 1985) and that were subsequently confirmed by electron (Casal et al., 1990) and immunofluorescent microscopy (Gonzalez et al., 1990). Similar spindle abnormalities were seen in mitosis in the larval neuroblasts of strong asp hypomorphs (Gonzalez et al., 1990). Typically these mitotic cells have long wavy arrays of microtubules. Hemi-spindles are frequently
observed in which a long dense array of microtubules is nucleated from a single centrosome.

Animals transheterozygous for particular mutant alleles of asp show an increased frequency of survival to adulthood (Gonzalez et al., 1990). However, survivors are female sterile and produce syncytial embryos displaying a number of abnormalities in nuclear divisions. One class of these embryos has no DNA, as judged by fluorescent staining, presumably reflecting failure of germline mitosis. A second class shows a variety of problems in nuclear division, including an abnormal ratio of centrosomes to nuclei corresponding to the polar regions of the spindle early in mitosis and the midbody at telophase.

Materials and Methods

Microcloning DNA from the asp Region

Chromosomal material corresponding to polytene map position 96A21-96B10 was microdissected from Oenothera third instar larval salivary gland polytene chromosomes. Two fragments were microdissected from two chromosomes and were pooled. The DNA was extracted and cloned, as described (Scalenghe et al., 1985; Saunders et al., 1989), in the lambda insert vector NM1149 (Murray, 1983). The recombinant clones were screened with labeled genomic DNA to identify clones containing repetitive DNA. Clones that appeared to be single-copy DNA were mapped by in situ hybridization as described (Shimell et al., 1991). Plasmid pMBO1367 contains an 18-kb SalI fragment from the chromosome walk to verify that they were derived from the asp region. A second chromosome microdissection was carried out using PCR amplification, as described previously (Saunders et al., 1989). In this case, two serial sections were performed through the 96A21-96B10 region of one chromosome. The amplifications yielded pools of DNA fragments of mean size 300 bp, as expected for Sau3A digestion. Microclone inserts and pools of DNA were labeled by random oligonucleotide priming and used to screen the cosmid libraries.

Chromosome Walking

Genomic clones were isolated from lambda libraries in Lambda dash (Stratagene, La Jolla, CA), and cosmid libraries were those constructed using the vectors Smar2 (Speck et al., 1988) or Loriste (Siden-Kiamos et al., 1990). The initial screening was carried out with cloned DNA arising from hybridization to the ends of the microcloning vectors. Periodically, the progress of the walk was monitored by in situ hybridization as described (Saunders et al., 1989). In situ hybridization of fragments from the chromosome walk to In(3R)Ub34-8ats chromosomes (with which the deficiency-bearing In(3R)Ub34-8ats was synthesized; Gonzalez et al., 1989) enabled the location of the distal breakpoint of the deficiency In(3R)Ub34-8ats to be determined. To map the distal breakpoint of Df(3R)H1b1, in situ hybridization with fluorescent labels (Saunders, 1994) was used to visualize signals derived from one or both homologues of 3R.

cDNAs were subsequently isolated by using segments of the walk to screen a testis cDNA library, kindly provided by T. Hazelrigg that had been constructed by a combination of random and oligo-dT priming in the vector AZAP and also embryonic cDNA libraries (provided by Brown, N.; Brown and Kafatos, 1988).

P element–mediated Rescue of asp1 Mutants

Rescue experiments were carried out using two Drosophila melanogaster lines containing homozygous second chromosome insertions of the P element transformation vector plasmids pMBO1366 and pMBO1367, respectively (Shimell et al., 1991). Plasmid pMBO1367 contains an 18-kb SalI fragment that includes both the tld and asp transcripts: pMBO1366 contains a 14-kb SalI fragment that includes the tld transcript but only the 5′ half of the asp transcript. Females with the genotype +; tld/asp; red1TM6B were crossed to 1367/1367; +/I; males. 1367/I; +; asp1 red1 males were selected from the F1 progeny and crossed to +/+; asp1 red1TM6B females to obtain asp1 red1/asp1 red1 individuals. 1367; asp1/asp1 animals did not show any morphological defect and were fully fertile, indicating that the 1367 insertion rescues the asp1 homozygous lethal phenotype. As a control, the same crosses were carried out using 1366 transformants. This insertion failed to rescue the asp1 mutation.

Sequence Determination and Analysis

Sequencing was carried out using the dideoxynucleotide chain termination procedure (Sambrook et al., 1989). Template clones for sequencing were produced by exonuclease III deletion (Sambrook et al., 1989) of insert subcloned in Bluescript SK+ and KS+. Sequences were assembled using Microgenie (Beckmann Instruments, Inc., Fullerton, CA) and LASergene (DNASTAR Inc., Madison, WI) software. Searches of GenBank/EMBL/DBJ database were conducted using BLAST with the MRC human genome mapping project computer. The protein sequence was analyzed using DOMAIN, SAPS, and COILS programs.

Expression of Asp Protein in Escherichia coli and Raising Polyclonal Antibodies

Plasmid pASP36 was constructed by inserting the Ncol–BamHI fragment from p6a (see Fig. 2) into the expression vector pET23d (Invitrogen Corp., San Diego, CA). Conditions for culture and induction were as described by the supplier. The polypeptide expressed by this clone represents the NH2-terminal portion of the Asp protein and does not include the putative actin-binding domain. This protein is insolubilized in vitro by preparing inclusion bodies, separating the proteins by polyacrylamide gel electrophoresis, excising the appropriate band from the gel, and electroeluting the protein as described by Leppard et al. (1983). Polyclonal sera were prepared by injecting rabbits as described (Harlow and Lane, 1988).

Embryo Staining

Embryos were fixed and stained as described by Gonzalez and Glover (1993). Propidium iodide was used to visualize DNA; the rat monoclonal antibody YL1/2 (Kilmartin et al., 1982) was used to stain microtubules, and the rabbit polyclonal antibody Rb3133 was used to stain the Asp protein. Staining was revealed using Texas red–conjugated goat anti–rat antibody and fluorescein-conjugated goat anti–rabbit antibody. All commercial antibodies were obtained from Jackson ImmunoResearch Labs, Inc. (West Grove, PA). Images were collected using an Optiphot microscope (Nikon Inc., Garden City, NY) with a confocal scanning head (model 600; BioRad, Richmond, CA) and merged using Adobe Photoshop V2.5.

Microtubule Preparation

Microtubules were purified from 0–3-h-old Drosophila embryos essentially as described by Goldenstein et al. (1986). Embryos were collected, dechorionated in 50% bleach, and washed with tap water and then with lysis buffer. About 3 ml of embryos were homogenized in 2 vol of ice cold lysis buffer (0.1 M Pipes/NaOH, pH 6.6, 5 mM EGTA, 1 mM MgSO4, 0.9 M glycerol, 1 mM DTT, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin) with a Dounce homogenizer. The microtubules were depolymerized by incubation on ice for 15 min, and the extract was then centrifuged at 16,000 g for 30 min at 4°C. The supernatant was recentrifuged at 135,000 g for 90 min at 4°C. Microtubules in this latter supernatant were polymerized by addition of GTP, to a final concentration of 1 mM, addition of taxol, to a final concentration of 20 mM, and incubation at room temperature for 30 min. 3-maliquots of extract was layered on top of 3-ml 15% sucrose cushions prepared in lysis buffer supplemented with 20 mM taxol and 1 mM GTP. After centrifuging at 54,000 g for 30 min at 20°C using a swing out rotor, the pellet was resuspended in lysis buffer containing taxol and GTP. To extract the MAPs, the concentration of
NaCl was adjusted to 0.4 M, and the samples were incubated for 30 min at 37°C. Microtubules were pelleted by centrifugation at top speed on a microcentrifuge for 30 min; the supernatant is considered to be the MAP fraction.

**Overlay Assays**

Proteins were blotted to PVDF membranes (Amersham Life Sciences, Pittsburgh, PA) after SDS-PAGE. Membranes were blocked with TBST (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 1 h, washed for 15 min in TBST plus 1% nonfat dry milk, and washed for 15 min in PEMF (0.1 mM Pipes/NaOH, pH 6.6, 1 mM EGTA, 1 mM MgSO_4, 25 mM NaF) buffer plus 1 mM GTP. The blots were then incubated overnight at room temperature with 5 μg/ml Drsophila tubulin (purified as described) in PEMF supplemented with 1 mM GTP and 20 mM taxol. The bound microtubules were detected by standard Western blot procedures using the monoclonal antibody Bx69 diluted five times.

**Results**

**Molecular Cloning of abnormal spindle**

Previous genetic mapping studies (Gonzalez et al., 1989) mapped asp to the cytological interval 96A21-96B10 between the distal breakpoint of In(3R)Ubx and the breakpoint of T(Y;3)B197 (Fig. 1). At the onset of this work there were no obvious molecular entry points into this region, and so we chose the technique of chromosome microdissection and microcloning to achieve this end. Two chromosomal segments corresponding to the asp interval were microdissected and DNA fragments extracted for cloning in the bacteriophage λ insertion vector λ1149. The chromosomal origins of these clones were confirmed by in situ hybridization, before using them to screen phage λ and cosm id genomic libraries (see Materials and Methods).

A resulting chromosome walk of 130 kb was carried out and clones correlated with the polyten chromosome map by in situ hybridization. The distal breakpoint of In(3R)Ubx and the breakpoint of T(Y;3)B197 (Fig. 1) and the proximal boundary of the region known to contain asp, was mapped to a 5.8-kb BamHI fragment at the extreme distal end of the walk. The distal end of the chromosome walk maps to the interband between 96A21-25 and 96B1-10. Subsequently, we found that a cytologically invisible deficiency Df(3R)Hd1 uncoveres not only the nearby zygotic embryonic lethal tolloid (tld), as reported by Shimell et al. (1991), but also asp.

To locate the asp gene, restriction fragments from the chromosome walk were used to screen a Drosophila testis cDNA library (a gift of Hazelrigg, T.). Genomic DNA corresponding to partial cDNA clones obtained from this screen was used in a secondary screen of an embryonic cDNA library (Brown and Kafatos, 1988). This yielded 6.5-kb cDNA clones corresponding to a transcription unit lying immediately distal to tld (Shimell et al., 1991). The tolloid62 mutation results from a P element insertion associated with a deletion of ~4 kb that removes the entire tld transcription unit and ~1 kb of the 5' end of the gene encoding the 6.5-kb transcript. Shimell et al. (1991) showed that tld can be rescued by two constructs, pMBO1366 and pMBO1367. Although pMBO1366 rescues the early embryonic zygotic lethality of tolloid62, the rescued flies exhibit cuticular defects. As such defects are associated with asp, it seemed that tolloid62 could be a tld asp double mutant. Consequently we tested whether these same transformants would rescue the recessive lethality of asp(+). pMBO1366 contains tld but only the 5' end of the candidate asp gene, whereas pMBO1367 contains both transcription units (see Materials and Methods; Fig. 1). We found that pMBO1367 rescues the lethality of asp(+) mutants while pMBO1366 does not. Taken together, these data confirm the identity of the 6.5-kb transcription unit as asp.

Furthermore, this gene has a developmental pattern of expression typical of many genes essential for the cell cycle whose products have to be maternally provided to the embryo. We used EcoRI–EcoRI or Neol–EcoRI fragments from the 6.5-kb cDNA clone p6a as hybridization probes on developmental Northern blots. One transcript of ~6.5 kb was readily observed (Fig. 2) in mRNA prepared from embryos and females (Fig. 2, lanes 1, 2, and 6) and was present at greatly reduced levels in larval and adult male mRNA (Fig. 2, lanes 3–5 and 7). When a SalI–NotI fragment from the plasmid pMBO1367, encompassing around 14 kb upstream of the NotI site on asp, was used as a probe, we observed the same 6.5-kb transcript plus one 3.8-kb transcript in embryonic mRNA corresponding to tld (Shimell et al., 1991) and a 5.2-kb transcript presumably corresponding to the recently described tolloid gene (Finelli et al., 1995; and data not shown).

**asp Encodes a Highly Basic Protein with Putative Actin and Calmodulin Binding Domains**

The 6.5-kb asp cDNA encodes a predicted polypeptide of 1,865-amino acid residues that has no homologues in the GenBank/EMBL/DDBJ database (Fig. 3 A). The asp protein is predominantly hydrophilic and strikingly basic, having a calculated pI of 10.8. Its secondary structure is predicted to be mostly α-helical. Analysis of the protein using the COILS program shows that short stretches of amino acids near the COOH terminus have the potential to form a coiled coil. There is a small sequence lying between residues 848 and 870 that has significant similarity to the core actin binding domain of a number of actin binding proteins, such as α-actinin (Noegel et al., 1987; Blanchard et al., 1989), fimbrin, spectrin, dystrophin (de Arruda et al., 1990; Matsudaira, 1991), and the Dictyostelium discoideum ABB120 (Bresnick et al. 1990; Fig. 3 B). These proteins either bundle actin filaments together (for example α-actinin) or attach actin filaments to other cellular structures. A second sequence lying between residues 938 and 968 corresponds to the conserved calmodulin binding (IQ) motif (Cheney and Mooseker, 1992) present in neuromodulin, a neuron-specific membrane-associated protein (Chapman et al., 1991); neurogranin, a neuron specific protein kinase C substrate (Baudier et al., 1991); the igloo gene product, a calmodulin-binding protein from the Drsophila central nervous system (Neel and Young, 1994); and in the “neck” regions of most forms of conventional and nonconventional myosin (for review see Cheney and Mooseker, 1992; Fig. 3 C). In addition Asp shows six consensus sites for phosphorylation by p34cdc2 and four consensus sites for phosphorylation by MAP kinase. Interestingly, these are all clustered in the NH2-terminal third of the molecule.
The asp<sup>1</sup> Allele Encodes a Truncated or Internally Deleted Protein

To further characterize the asp gene product, we raised rabbit polyclonal antibodies to a truncated Asp protein expressed in E. coli. The NcoI–BamHI fragment of cDNA 6a (Fig. 4) was subcloned into the expression vector pET23d. The resulting construct, pASP36, expresses a polypeptide corresponding to amino acid residues 1–512, with a predicted molecular weight of 55.9 kD. This polypeptide does not contain the putative actin binding domain of the Asp protein.

The resulting serum Rb3133 recognizes one polypeptide of ~220 kD in immunoblots of third instar larval brains (Fig. 5). To confirm that Rb3133 reacts specifically with the Asp protein, brains from larvae of the transgenic strain 1366 (see Materials and Methods) were used in Western blot analysis (Fig. 5, lane 2). Flies transformed with pMBO1366 are expected to synthesize two forms of the Asp protein: full length protein of 220 kD, derived from the endogenous copy of asp at 96A and a truncated form derived from the transgene. As expected, the serum detects the 220-kD wild-type asp protein plus a 124-kD polypeptide corresponding to the COOH-terminal truncated form of the asp protein encoded by plasmid pMBO1366.

When extracts from brains of asp<sup>1</sup> homozygous larvae were analyzed (Fig. 5, lane 3), the 220-kD protein is no longer observed, but the serum labels a polypeptide of ~130 kD. As the polyclonal antibodies were raised against the NH<sub>2</sub> terminus of the Asp protein, it seems that the asp<sup>1</sup> mutation results in a COOH terminus truncation or in an internal deletion of the Asp protein.
The Asp Protein Associates with Microtubules

As mutations in *asp* affect the behavior of spindle microtubules, we sought to determine whether the Asp protein was itself microtubule associated. We purified microtubules from *Drosophila* embryos and took aliquots at each stage of the purification for electrophoresis and blotting onto PVDF membranes. These blots were incubated with the polyclonal serum Rb3133 and with the monoclonal antibody Bx69, which detect the Asp protein and \( \beta \)-tubulin, respectively (Fig. 6, A and B). Enrichment in tubulin is paralleled by an enrichment in the Asp protein. Moreover, the Asp protein seems to bind microtubules with a very high affinity as it is not released from the microtubule pellet by salt conditions that are known to dissociate most MAPs (Fig. 6, lanes 6). Asp protein is also not liberated after incubation with ATP (data not shown).

We further assessed the ability of Asp protein to bind microtubules in overlay assays in which bacterially expressed Asp fragments were separated by SDS-PAGE and blotted to a membrane that was subsequently incubated with microtubules. Bound microtubules were then detected by probing with the monoclonal anti--\( \beta \)-tubulin antibody Bx69. These assays were performed using either total *E. coli* proteins from cells expressing Asp11 and Asp36 (Fig. 7, A and B) or the corresponding purified fusion proteins (Fig. 7 C). Microtubules were observed to bind to both Asp36 and Asp11, but binding to Asp36 appears to be of a higher affinity (Fig. 7, B and C). Binding seems to be specific, as no detectable binding of microtubules to total *E. coli* proteins was observed in strains that had not been induced to express the fusion proteins (Fig. 7, A and B). To ensure that differences in binding did not simply reflect differences in the degree of induction of the two fusion proteins in the bacteria, we repeated the microtubule binding assay using increasing amounts of the two purified fusion proteins (Fig. 7 C). Differences in the binding affinity can be seen at all protein concentrations; Asp11 binding to microtubules was barely detectable when 1.6 \( \mu \)g of protein are used, whereas microtubule binding to Asp36 is readily detectable when 0.4 \( \mu \)g of protein were blotted to the membrane. This shows that the NH\(_2\)-terminal third of the Asp protein binds more avidly to microtubules than does the central part of the molecule.

The Asp Protein Associates with the Mitotic Spindle

Mutations in *asp* affect the morphology of both the mitotic spindle at several developmental stages and the meiotic spindle. At all stages mutant spindle microtubules may be...
described as having a long and wavy appearance, and it is
not uncommon to see the loss of bipolarity in the form of
hemispindle structures. To determine whether Asp pro-	ein is a constituent of the wild-type spindle we used the
Rb3133 antibody to localize the Asp protein with respect
to microtubules in mitosis in syncytial embryos (Fig. 8).
During interphase, Asp protein appears to be distributed
throughout the cytoplasm (Fig. 8a), but as the syncytium
enters mitosis and the bipolar spindle is formed, Asp is
seen in association with the polar regions (Fig. 8b). This
polar association becomes tighter throughout metaphase
and anaphase (Fig. 8c and d), but at telophase, as the
chromatin is decondensing and the spindle begins to disas-
semble, the Asp protein appears to move away from the
region occupied by the centrosome onto the central region
of the spindle microtubules.

Discussion

Several pieces of evidence confirm the molecular identity
of the asp gene. The full length gene rescues asp in germ-
line transformants, whereas a truncated gene does not.
Furthermore, antibodies raised against a segment of the
gene expressed in E. coli recognize a truncated form of Asp
protein in asp1 homozygotes. These same antibodies have
allowed us to examine the subcellular distribution of the
Asp protein in cytological preparations by immunostaining
and biochemically after subcellular fractionation. These
experiments show the Asp protein to copurify with micro-
tubules and that it remains associated with the microtubules in salt
concentrations that remove many other MAPs. That the
Asp protein is a MAP is confirmed by its association with
the polar regions of spindles in mitosis.

Asp is the first metazoan MAP to be identified through
a genetic approach, and it is gratifying that the naming of
the gene by Ripoll et al. (1985) was predictive of the local-
ization and function of the protein. The protein is highly

Figure 4. Asp expression constructs. The upper portion of the
figure shows the asp cDNA indicating restriction cleavage sites
and localization of the putative actin and calmodulin binding
sites. Segments of the protein expressed in E. coli are indicated
by shaded bars. The truncated protein expressed in flies trans-
formed by pMBO1366 is indicated by a solid line.

Figure 5. Shortened proteins are produced by the asp1 mutant
and by pMBO1366 transformants. Western blot analysis of asp
expression using the antibody Rb3133. (Lane 1) Proteins from 8
wild-type larval brains; (lane 2) proteins from 8 brains of larvae
transformed by pMBO1366; (lane 3) proteins from 12 asp/asp1
larval brains.

Figure 6. Asp copurifies with microtubules. A shows a Western
blot fractionated by 7.5% SDS-PAGE. B shows the same protein
preparations on a Coomassie blue–stained 10% SDS-PAGE. (A
and B) Microtubule purification from 0–3-h-old Drosophila em-
byros after Taxol-induced polymerization. Asp was detected us-
ring the Rb3133 antibody and tubulin by the Bx69 antibody. Sam-
ple are as follows: (lane 1) 20 μg of crude embryonic protein
extract; (lane 2) 20 μg of protein after the 16,000 g centrifugation;
(lane 3) 20 μg of protein from the supernatant fraction after sur-
crome gradient centrifugation; (lane 4) 10 μg of the microtubules
and associated proteins; (lane 5) 5 μg of the final microtubule
preparation; (lane 6) 10 μg of the final MAP preparation.
alone is more resistant to certain depolymerizing drugs of microtubules in the presence of the promoter domain organize them into clusters of spirals. Moreover, assembly domain and find that the residual promoter domain differs gested MAP2 with chymotrypsin to remove the projection bly, and a “projection” domain thought to interact with domain, which binds to microtubules to promote assembly of microtubules leading to net microtubule growth over, in vitro studies show that MAP2 can decrease the but has recently been shown to be present in rat testes firmed. MAP2, for example, is predominantly found in brain teons and dendrites of neurons. In some cases, these pro- teins associated with the stable microtubules of the ax- somes. MAPs could be functionally redundant. A 230-kD protein, XMAP 230, has been purified from Xenopus oocytes and found to be present in all dividing cells (Andersen et al., 1995). It is localized to microtubules in interphase but dissociates from them upon entry into mitosis, to reassociate later in the mitotic cycle. It is phosphorylated by mitotic extracts whereupon it has a reduced affinity for microtu- bules. Upon binding it increases microtubule growth rate, decreases the rate of shrinking, and suppresses microtu- bule loss by “catastrophe.” It thus stabilizes interphase microtubules and can locally modify microtubule behavior during mitosis. As the Asp protein has numerous sites for phosphorylation by p34cdc2 and mitogen activated protein kinase, it is not inconceivable that cyclical phosphorylation could affect its properties throughout the mitotic cycle.

The presence of wild-type Asp protein in the polar regions of the spindle points to this being the site of its action and suggests that it may play a crucial role in regulating the dramatic changes of microtubule dynamics upon the entry into mitosis. Its presence in the astral microtu- bules may be important to mediate interactions between microtubules and the actin cytoskeleton. This function is suggested by the actin-binding motif in the central domain of the protein. This motif is homologous to ones seen in a number of actin bundling or gelation factors. These include α-actinin (Noegel et al., 1987; Blanchard et al., 1989), a filamenous actin cross-linking protein found in stress fibers and adhesion plaques in nonmuscle cells and in Z-discs in muscle cells; it has a single actin binding motif and can crosslink actin filaments into a homodimer. Single actin basic, and in its central region lies a putative actin-binding motif. Basic regions within other MAPs play a significant part in conferring the ability to bind microtubules. The Asp protein is no exception to this rule in that at least one segment of this basic molecule, a polypeptide comprised of the 512 NH2-terminal amino acids, can be shown to bind microtubules in vitro. The central region of the protein binds microtubules poorly. Unfortunately, we have been unable to test the microtubule binding ability of the COOH-terminal region, as this segment of the protein appears unstable in both bacterial and insect cell expression systems.

It is instructive to compare the domain organization and microtubule binding properties of Asp with other MAPs that have been characterized. These are principally of mammalian origin, and most have been purified from brain as proteins associated with the stable microtubules of the axons and dendrites of neurons. In some cases, these proteins may be expressed specifically in brain. However, in many cases their presence in other tissues is being con- nected by phosphorylation with p34cdc2 kinase, mitogen ac- tivated protein kinase, or the NIMA mitotic kinase. In contrast, phosphorylation of MAP4 by p34cdc2 kinase abolishes its microtubule stabilizing activity, interestingly without altering its ability to bind microtubules (Ookata et al., 1995). MAP4 is the major MAP of many mammalian cell lines and is present along the length of both interphase and mitotic microtubules (Bulinski and Borisy, 1980; De- Brabander et al., 1981). There is evidence that MAP4 mediates the association of cyclin B with microtubules in both the starfish oocyte and in cultured mammalian cells (Ookata et al., 1992, 1993, 1995). It may therefore provide a general mechanism for destabilizing interphase microtu- bules upon entry into mitosis. Other MAPs could also have such a role, and as with the kinesin-like proteins, MAPs could be functionally redundant. A 230-kD protein, XMAP 230, has been purified from Xenopus oocytes and found to be present in all dividing cells (Andersen et al., 1995). It is localized to microtubules in interphase but dissociates from them upon entry into mitosis, to reassociate later in the mitotic cycle. It is phosphorylated by mitotic extracts whereupon it has a reduced affinity for microtu- bules. Upon binding it increases microtubule growth rate, decreases the rate of shrinking, and suppresses microtu- bule loss by “catastrophe.” It thus stabilizes interphase microtubules and can locally modify microtubule behavior during mitosis. As the Asp protein has numerous sites for phosphorylation by p34cdc2 and mitogen activated protein kinase, it is not inconceivable that cyclical phosphorylation could affect its properties throughout the mitotic cycle.

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Figure 8. Immunolocalization of Asp in the mitotic cycle in syncytial embryos. Simultaneous staining for DNA with propidium iodide and tubulin with YL1/2 primary antibody and rhodamine-conjugated goat anti–rat IgG are shown in the first column and subsequent red channel of the merged image. Staining of Asp using Rb3133 primary antibody and FITC-conjugated goat anti–rabbit IgG is shown in the middle column and subsequent green channel in the merged image. The mitotic phases are (a) interphase, (b) prophase, (c) metaphase, (d) anaphase, and (e) telophase. The scale bar refers to the main set of panels. Single mitotic figures have been selected for the inset at a fourfold greater magnification. Bar, 25 μm.
binding motifs are also found on dystrophin and spectrin (de Arruda et al., 1990; Matsudaira, 1991). Fimbrin, an actin-associated protein found in microvilli and filopodia, on the other hand, has a duplicated actin binding motif that enables it to bundle actin filaments as a monomer (de Arruda et al., 1990). It has homology to plastins, cytoplasmic actin binding proteins, and the protein encoded by the yeast gene ABP7 or Sac6p (Adams et al., 1995). The association of microtubule and microfilament networks is nowhere more obvious than in the vicinity of centrosomally nucleated asters. In the syncytial blastoderm Drosophila embryo, for example, the actin caps that form above interphase nuclei are positioned by the centrosome and its associated microtubules. Moreover, the spatial coordination of mitosis and cytokinesis requires the contractile actin ring that will dictate the cleavage furrow to be correctly positioned with respect to the spindle. Classical experiments with echinoderm eggs showed that moving the spindle by micromanipulation resulted in the corresponding repositioning of the cleavage furrow. However, the experimental manipulation of such embryos to produce two mitotic spindles within a single cell led to the generation of a third cleavage furrow between the asters of the juxtaposed spindles (for review see Rappaport, 1986a,b). The nature of the molecular “signal” that positions the actin ring with respect to the asters is unknown, but it is possible that a molecule such as the Asp protein could fulfill this role.

The presence of a potential calmodulin binding site in the central region of the molecule implies that the Asp protein may be responsive to fluxes in calcium ions known to occur in the polar regions of the spindle. Preliminary experiments show that the motif present in Asp enables it to bind to calmodulin in a Ca\textsuperscript{2+}-dependent manner (data not shown). Calmodulin is a member of the EF hand superfamily of proteins. There is strong evidence for a direct role for two members of this family in spindle pole body function in budding yeast. Calmodulin itself has been shown by both genetic and molecular studies (Geiser et al., 1993; Stirling et al., 1994, 1996; Sundberg et al., 1996) to be essential for spindle function as a result of its interaction with the COOH terminus of Spc110p, an essential protein with a large coiled-coil domain that provides a spacer between the central and inner plaques of the SPB (Kilmartin, 1993). The second EF hand protein localized to the SPB is the product of the CDC31 gene, which associates with the KARI gene product to form a complex (Spang et al., 1993, 1995; Biggens and Rose, 1994; Vallen et al., 1994). Roles for these proteins in spindle function are likely to be conserved. Calmodulin is the major intracellular Ca\textsuperscript{2+} receptor and is involved in many cellular processes; it is known to be localized to the microtubules of metazoan spindles (e.g., Stemple et al., 1988), and it appears to be important in regulating the function of its dependent kinase and phosphatase in the metaphase–anaphase transition and in spindle behavior (Morin et al., 1994; Yoshida et al., 1994). CDC31 is the yeast homologue of centrin, a protein first identified in the Chlamydomonas basal body and subsequently found to be a universal component of centriomes in animal cells (for review see Salisbury, 1995). There are therefore a large number of potential ways in which Asp protein function might be mediated through an association with one of these EF hand proteins.

It is evident that many questions are thrown open by our finding that the Asp protein is a MAP containing potential actin and calmodulin binding motifs. However, our work has now generated the molecular tools with which we can begin to address some of the above issues.

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