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^1 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_2-terminal 512-amino acid peptide, which has a number of potential phosphorylation sites for $p34^{cdc2}$ and MAP kinases, strongly binds to microtubules. The central 579-amino acid segment of the molecule contains one short motif homologous to sequences in a number 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*)[1] 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^+ 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^1 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

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1. *Abbreviations used in this paper*: *asp*, *abnormal spindle*; spb, spindle pole body; *tld*, *tolloid.*

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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 and broadened or fused spindles with wavy microtubules.

Taken together, the various phenotypes seen in *asp* homozygotes suggest that the *asp* gene product may be involved in some aspect of spindle microtubule dynamics. In this paper we show that *asp* encodes a microtubule-associated protein that localizes 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 Oregon R 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 insertion 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 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 *Sau*3A 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 Smart2 (Speek et al., 1988) or Lorist6 (Siden-Kiamos et al., 1990). The initial screening was carried out with cloned DNA arising from the first microcloning experiment. Subsequent steps of the walk used terminal RNA probes synthesized from the promoters at the ends of the cloning 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+3L)ats* chromosomes (with which the deficiency-bearing $In(3R)Ubx^{7LL}ats^{R}$ was synthesized; Gonzalez et al., 1989) enabled the location of the distal breakpoint of the deficiency $In(3R)Ubx^{7LL}ats^{R}$ to be determined. To map the distal breakpoint of $Df(3R)Hd^{\gamma 1}$, 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 λZAP and also embryonic cDNA libraries (provided by Brown, N.; Brown and Kafatos, 1988).

P element–mediated Rescue of asp^1 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 +/+; asp^1 *red*/TM6B were crossed to 1367/1367; +/+ males. 1367/+; asp^1 *red*/+ males were selected from the F1 progeny and crossed to +/ +; asp^1 *red*/TM6B females to obtain asp^1 *red*/asp^1 *red* individuals. 1367; asp^1/asp^1 animals did not show any morphological defect and were fully fertile, indicating that the 1367 insertion rescues the asp^1 homozygous lethal phenotype. As a control, the same crosses were carried out using 1366 transformants. This insertion failed to rescue the asp^1 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 inserts 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/DDBJ 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 NcoI–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 NH_2-terminal portion of the Asp protein and does not include the putative actin binding domain. This polypeptide was insoluble and was purified 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).

Plasmid pASP11 was constructed by inserting the BamHI–EcoRI fragment from p6a (see Fig. 2) into the expression vector pQE31 (Qiagen, Inc., Santa Clara, CA). Conditions for culture, induction, and denaturing purification on Ni–agarose columns (Qiagen, Inc.) were as described by the supplier.

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 Goldstein 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 $MgSO_4$, 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-ml aliquots 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 *Drosophila* 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 $In(3R)Ubx^{7LL}ats^{R}$ 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 cosmid genomic libraries (see Materials and Methods). A resulting chromosome walk of 130 kb was carried out and clones correlated with the polytene chromosome map by in situ hybridization. The distal breakpoint of $In(3R)Ubx^{7LL}ats^{R}$, which does not uncover *asp* and defines the proximal boundary of the region known to contain *asp*, was mapped to a 5.8-kb BamHI fragment at the extreme proximal 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 $Df(3R)Hd^{\gamma 1}$ uncovers 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 tld^{68-62} 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 tld^{68-62}, the rescued flies exhibit cuticular defects. As such defects are associated with *asp*, it seemed that tld^{68-62} could be a *tld asp* double mutant. Consequently we tested whether these same transformants would rescue the recessive lethality of asp^{1}. 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 the pMBO1367 rescues the lethality of asp^{1} 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 NcoI–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*) but 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 *tolkin* 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 1,863–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* ABP120 (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 *Drosophila* 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 $p34^{cdc2}$ and four consensus sites for phosphorylation by MAP kinase. Interestingly, these are all clustered in the NH_2-terminal third of the molecule.

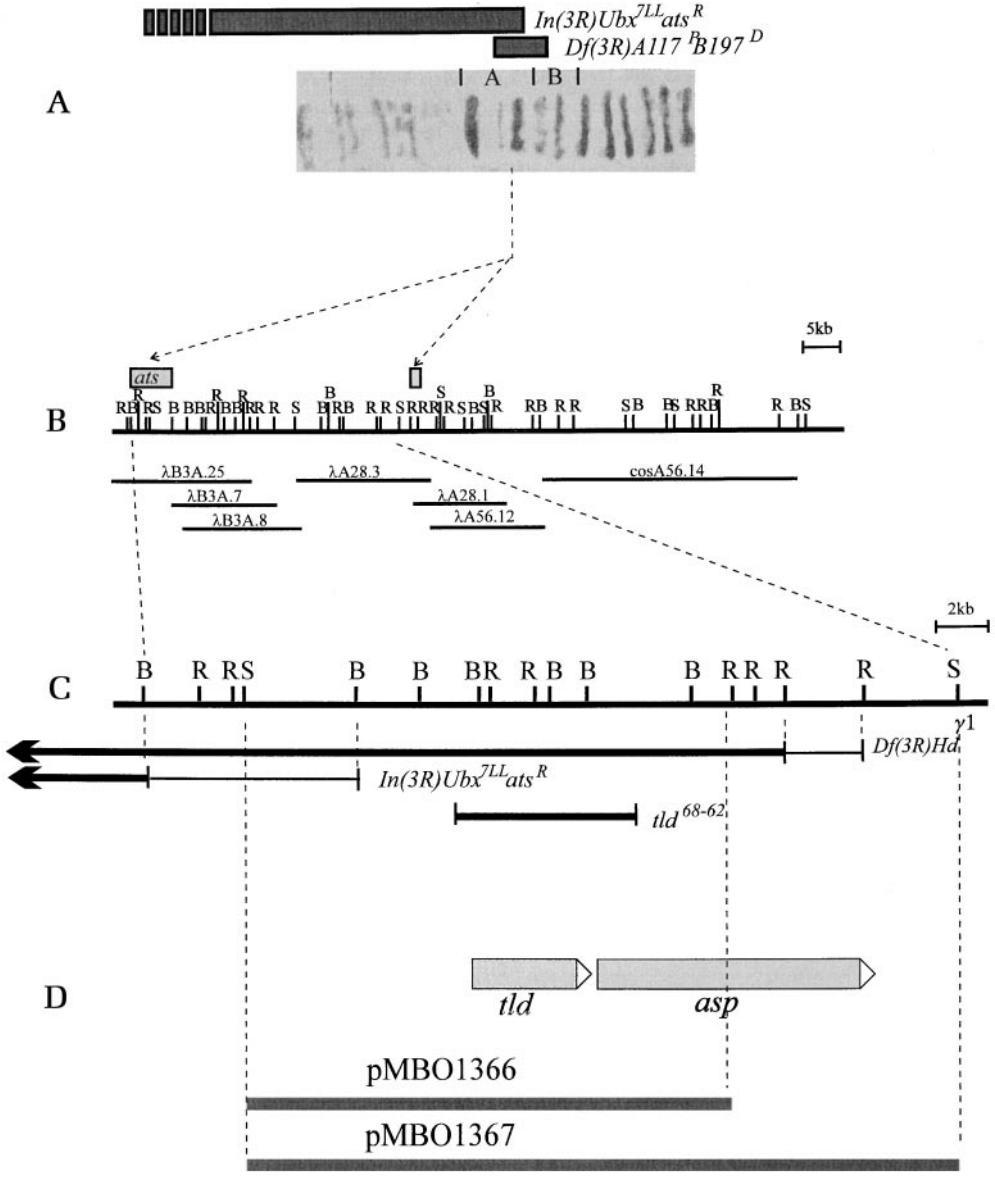

Figure 1. A cytogenetic and molecular map of the *asp* region. *A* is a representation of divisions 95 and 96 of the polytene map of chromosome arm 3R. Subdivisions A and B are indicated. The rearrangements used by Gonzalez et al. (1989) to define the *asp* region, $In(3R)Ubx^{7LL}ats^{R}$ and $Df(3R)A117^{P}B197^{D}$ are indicated above the chromosomes. *B* shows the proximal section of the chromosome walk linked to the cytological map. B, R, and S represent BamHI, EcoRI, and SalI restriction sites, respectively. Horizontal lines below the restriction map indicate phage lambda and cosmid clones isolated from genomic libraries. Boxed fragments indicate fragments mapped by in situ hybridization. One contains the distal breakpoint of $In(3R)Ubx^{7LL}ats^{R}$ (*ats*), while the other is an EcoRI fragment recovered by microcloning. *C* is an expansion of the proximal section of the chromosome walk. The distal breakpoints of $In(3R)Ubx^{7LL}ats^{R}$ and $Df(3R)Hd^{\gamma 1}$ are indicated. Uncertainties about the cytological limits of the deficiencies are indicated by the thinner lines. The limits of the deletion associated with the P element insertion *tld* allele tld^{68-62} are indicated by the bar. *D* illustrates the transcription units of *tld* and *asp* as well as the two fragments used in P element mediated rescue experiments. pMBO1367 contains an 18-kb SalI fragment containing both *tld* and *asp*. pMBO1366 contains a 14-kb SalI fragment containing *tld* but only the 5′ portion of *asp*.

The asp^1 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^1 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_2 terminus of the Asp protein, it seems that the asp^1 mutation results in a COOH terminus truncation or in an internal deletion of the Asp protein.

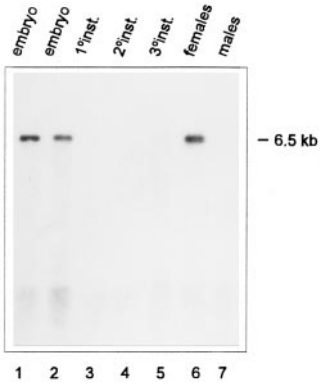

Figure 2. Developmental pattern of expression of *asp* and its flanking transcripts. Northern blot analysis of *asp* expression (*asp* transcript is ~6.5 kb). An Nco1–EcoRI fragment from the 6.5-kb *asp* cDNA clone was used as the probe. Poly A+ is from 0–3 h-old embryos (lane *1*), 3–6 h-old embryos (lane *2*), first instar larvae (lane *3*), second instar larvae (lane *4*), third instar larvae (lane *5*), adult females (lane *6*), and adult males (lane *7*).

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 β-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–β-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 μg of protein are used, whereas microtubule binding to Asp36 is readily detectable when 0.4 μg 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.

A.

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MELVWSPVLEVACKETLQLIDNRNFRKEVMIILKSKSNQPVKNPRKFPTVGKTLQLKSPT      60
GAGKTMKSVVSAAVQQKKRMSAAAAPPSKQTWRVTAPSRPAAWAHPPPQAPLVEKNVYKT     120
PQEEPVYISPQPRSLKENLSPMTPGNLLDVIDNLRFTPLTETRGKGQATIFPDNLAAWPT     180
PTLKGNVKSCANDMRPRRITPDDLEDQPATNKTFDVKHSETINISLDTLDCSRIDGQPHT     240
PLNKTTTIVHATHTRALACIHEEEGHSPPRTPTKSAIHDLKRDIKLVGSPLRKYSESMKD     300
LSLLSPQTKYAIQGSMPNLNEMKIRSIEQNRYYQEQQIQIKAKDLNSSSSSEASLAGQQE     360
FLFNHSEILAQSSRFNLHEVGRKSVKGSPVRNPHKRRSHELSFSDAPSNESLYRNETVAI     420
SPPKKQRVEDTTLPRSAAPANASARSSSAHAWPHAQSKKFKLAQTMSLMKKPATPRRVRD     480
TSIQPSVKLYDSELYMQTCINPDPFAATTTIDPFLASTMYLDEQAVDRHQADFKKWLNAL     540
VSIPADLDADLNNKIDVGKLFNEVRNKELVVAPTKEEQSMNYLTKYRLETLRKAAVELFF     600
SEQMRLPCSKVAVYVNKQALRIRSDRNLHLDVVMQRTILELLLCFNPLWLRLGLEVVFGE     660
KIQMQSNRDIVGLSTFILNRLFRNKCEEQRYSKAYTLTEEYAETIKKHSLQKILFLLPFL     720
DQAKQKRIVKHNPCLFVKKSPHKETKDILLRFSSELLANIGDITRELRRLGYVLQHRQTF     780
LDEFDYAFNNLAVDLRDGVRLTRVVEVILLRDDLTRQLRVPAISRLQRIFNVKLALGALG     840
EANFQLGGDIAAQDIVDGHREKTLSLLWQIIYKFRSPKFHAAATVLQKWWRRHWLHVVIQ     900
RRIRHKELMQRRAAAIVIQAVVRGHQARKSVKKLTERTQAAIILQKFTRRYLAQKQLYQ     960
SYHSIITIQRWWRAQQLGRQHRQRFVELREAAIFLQRIWRRRLFAKKLLAAAETARLQRS    1020
QKQQAAASYIQMQWRTYQLGRIQRHEFLRQRDLIMFVQRRMRSKWSMLEQRKEFQQLKRA    1080
AINIQQRWRAKLSMRKCNADYLALRSSVLKVQAYRKATIQMRIDRNHYYSLRKNVICLQQ    1140
RLRAIMKMREQRENYLRLRNASILVQKRYRMRQQMIQDRNAYLRTRKCIINVQRRWRATL    1200
QMRRERKNYLHLQTTTKRIQIKFRAKREMKKQRAEFLQLKKVTLVVQKRRRALLQMRKER    1260
QEYLHLREVTIKLQRRFHAQKSMRFMRAKYRGTQAAVSCLQMHWRNHLLKRERNSFLQL    1320
RQAAITLQRRYRARLNMIKQLKSYAQLKQAAITIQTRYRAKKAMQKQVVLYQKQREAIIK    1380
VQRRYRGNLEMRKQIEVYQKQRQAVIRLQKWWRSIRDMRLCKAGYRRIRLSSLSIQRKWR    1440
ATVQARRQREIFLSTIRKVRLMQAFIRATLLMRQQRREFEMKRRAAVVIQRRFRARCAML    1500
KARQDYQLIQSSVILVQRKFRANRSMKQARQEFVQLRTIAVHLQQKFRGKRLMIEQRNCF    1560
QLLRCSMPGFQARARGFMARKRFQALMTPEMMDLIRQKRAAKVIQRYWRGYLIRRRQKHQ    1620
GLLDIRKRIAQLRQEAKAVNSVRCKVQEAVRFLRGRFIASDALAVLSQLDRLSRTVPHLL    1680
MWCSEFMSTFCYGIMAQAIRSEVDKQLIERCSRIILNLARYNSTTVNTFQEGGLVTIAQM    1740
LLRWCDKDSEIFNTLCTLIWVFAHCFKKRKIIHDYMTNPEAIYMVRETKKLVARKEKMKQ    1800
NARKPPPMTSGRYKSQKINFTPCSLPSLEPDFGIIRYSPYTFISSVYAFDTILCKLQIDMF.    1862
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p34^{cdc2} consensus phosphorylation sites
bold MAP Kinase consensus phosphorylation sites
– putative actin binding site
– putative calmodulin binding site

B.

Putative Actin Binding Site

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IAAQDIVDGHREKTLSLLWQLIYKF   Asp
IGAEELVDGNLKMTLGMIWTIILRF   α-Actinin (Drosophila melanogaster)
IGAEDIVDGNPRLILGLIWTIILRF   Spectrin beta-heavy chain (Drosophila melanogaster)
IGGQDLNEGNRTLTLALIWQLMRRY   L-Plastin (human)
IGAEDIVDSQLKLILGLIWTLILR    Actin-binding protein 120 (Dictyostelium discoideum)
IGSSDIVDGNHKLTLGLIWNII      Dystrophin (cnicken muscle)
IESSDIVDGNKLLTLGLVWQLM      Fimbrin (S. cerevisiae)
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C.

Putative Calmodulin Binding Site

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RRHRAATVIQAVFRGHQMRKYVKLFK  Asp
KAHKAATKIQASFRGHITRKKLKGEK  Neuromodulin (bovine)
GANAAAAKIQASFRGHMARKKIKSGE  Neurogranin (rat)
DEAKAATKIQAVFRGHKVRETMKKSE  igloo (V-module) (Drosophila melanogaster)
RMRRAAITIQRYVRGHQARCYATFLR  Dilute Myosin heavy chain (chicken)
MKVNCATLLQAAYRGHSIRANVFSVL  Myosin 2 (Saccharomyces cerevisiae)
RVAELATLIQKKFRGWCCRKRYQLMR  Brush border myosin I (chicken)
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Figure 3. (*A*) The sequence of the Asp protein. Consensus sites for phosphorylation by p34^{cdc2} and MAP kinases are shown. Also shown are the putative actin and calmodulin binding sites. (*B*) Comparison of actin binding motifs. Comparison between Asp putative actin binding site and actin binding sites present in α-actinin, spectrin, plastin, ABP120, dystrophin, and fimbrin. Only a selection of these actin binding domains is shown. Identical residues to Asp are shaded. (*C*) Comparison of calmodulin binding motifs. A comparison between the Asp putative calmodulin binding site and the calmodulin binding sites present in a number of other proteins. This list is not comprehensive. Identical residues have been shaded.

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

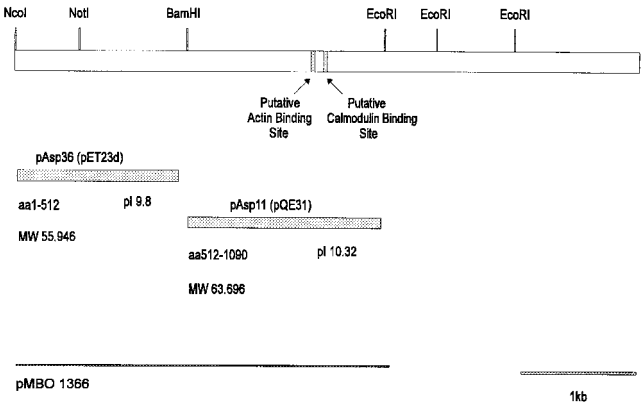

Figure 4. Asp expression constructs. The upper portion of the figure shows the *asp* cDNA indicating restriction cleavage sites and localization of the putative actin and calmodulin binding sites. Segments of the protein expressed in *E. coli* are indicated by shaded bars. The truncated protein expressed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

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 protein 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. 8 *a*), but as the syncytium enters mitosis and the bipolar spindle is formed, Asp is seen in association with the polar regions (Fig. 8 *b*). This polar association becomes tighter throughout metaphase and anaphase (Fig. 8, *c* and *d*), but at telophase, as the chromatin is decondensing and the spindle begins to disassemble, 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 germline 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 asp^1 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 microtubules after Taxol-induced tubulin polymerization 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 localization and function of the protein. The protein is highly

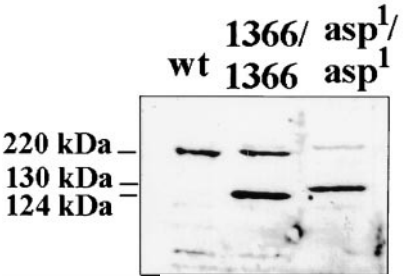

Figure 5. Shortened proteins are produced by the asp^1 mutant and by pMBO1366 transformants. Western blot analysis of asp expression using the antibody Rb3133. (Lane *1*) Proteins from 8 wild-type larval brains; (lane *2*) proteins from 8 brains of larvae transformed by pMBO1366; (lane *3*) proteins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

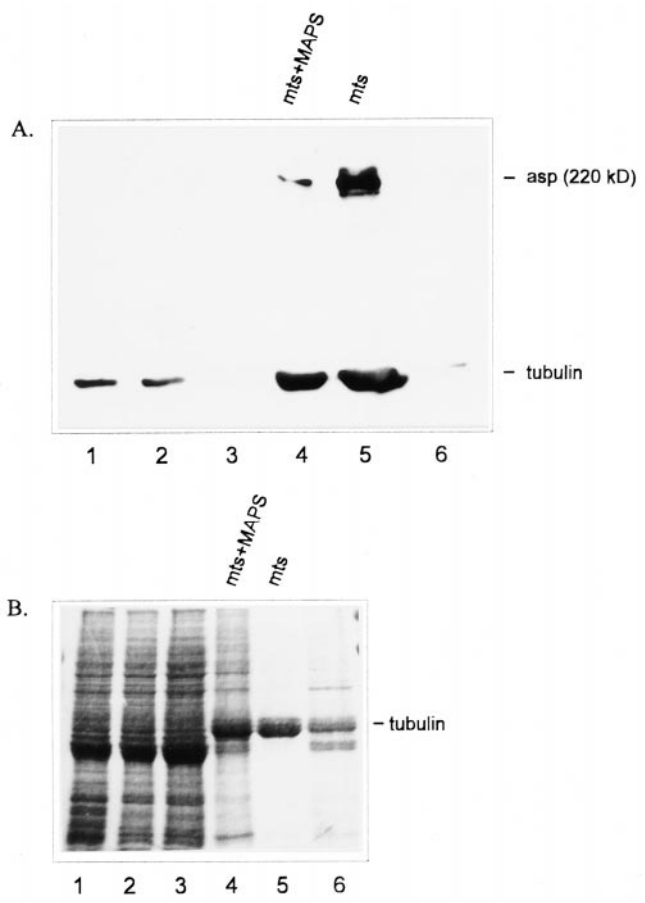

Figure 6. Asp copurifies with microtubules. *A* shows a Western blot fractionated by 7.5% SDS-PAGE. *B* shows the same protein preparations on a Coomassie blue–stained 10% SDS-PAGE. (*A* and *B*) Microtubule purification from 0–3-h-old *Drosophila* embryos after Taxol-induced polymerization. Asp was detected using the Rb3133 antibody and tubulin by the Bx69 antibody. Samples are as follows: (lane *1*) 20 μg of crude embryonic protein extract; (lane *2*) 20 μg of pellet after the 16,000 *g* centrifugation; (lane *3*) 20 μg of protein from the supernatant fraction after sucrose 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.

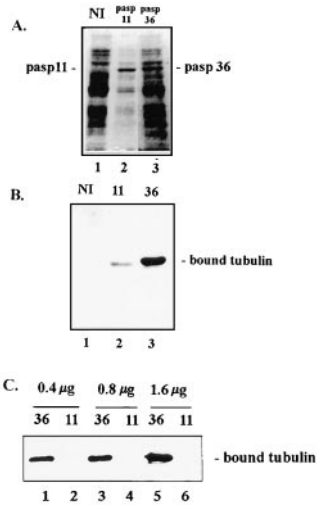

Figure 7. The NH_2-terminal segment of Asp binds polymerized tubulin in vitro. (*A*) SDS-PAGE of total proteins from noninduced *E. coli* strain carrying pAsp11 (lane *1*), *E. coli* pAsp 11 induced to express (lane *2*), and *E. coli* pAsp36 induced to express (lane *3*). An overlay of such a gel is shown in *B*, in which the blotted proteins have been overlaid with polymerized tubulin that is subsequently detected with the Bx69 monoclonal anti–β-tubulin (see Materials and Methods). *C* shows an overlay assay carried out on the indicated amounts of purified Asp segment after expression in *E. coli.*

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 NH_2-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 confirmed. MAP2, for example, is predominantly found in brain but has recently been shown to be present in rat testes (Loveland et al., 1996). In general, MAPs bind to microtubules to increase their stability. Studies of MAP2C in vivo, for example, show that it bundles and stabilizes microtubules in cultured fibroblasts (Umeyama et al., 1993). Moreover, in vitro studies show that MAP2 can decrease the frequency of transitions between the growth and shortening of microtubules leading to net microtubule growth (Kowalski and Williams, 1993; Itoh and Hotani, 1994). Like several other MAPs, MAP2 has a short "promoter" domain, which binds to microtubules to promote assembly, and a "projection" domain thought to interact with other cytoskeletal elements. Fellous et al. (1994) have digested MAP2 with chymotrypsin to remove the projection domain and find that the residual promoter domain differs from the full length protein in that it binds microtubules to organize them into clusters of spirals. Moreover, assembly of microtubules in the presence of the promoter domain alone is more resistant to certain depolymerizing drugs than assembly in the presence of the full length protein. Thus the projection domain would appear to modify the interactions of MAP2 with microtubules in their assembly. The Asp protein may be seen as being structurally similar and functionally analogous, having an NH_2-terminal microtubule binding domain and central and COOH-terminal regions with the potential to interact with other cytoskeletal components.

It may be not only that microtubule dynamics are affected by the *asp* mutation but possible that the function of other MAPs, in particular motor molecules, could be impaired if the mutant protein can still bind microtubules. The binding of MAP2, for example, is known to inhibit the motility of kinesin and cytoplasmic dynein, but this is reversed by phosphorylation of the protein (Lopez and Sheetz, 1995). This specifically requires a cAMP-dependent kinase that copurifies with MAP2, and it is not affected by phosphorylation with $p34^{cdc2}$ kinase, mitogen activated protein kinase, or the NIMA mitotic kinase. In contrast, phosphorylation of MAP4 by $p34^{cdc2}$ 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; DeBrabander 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 microtubules 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 microtubules. Upon binding it increases microtubule growth rate, decreases the rate of shrinking, and suppresses microtubule 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 $p34^{cdc2}$ 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 microtubules 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 filamentous 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 cross link actin filaments into a homodimer. Single actin

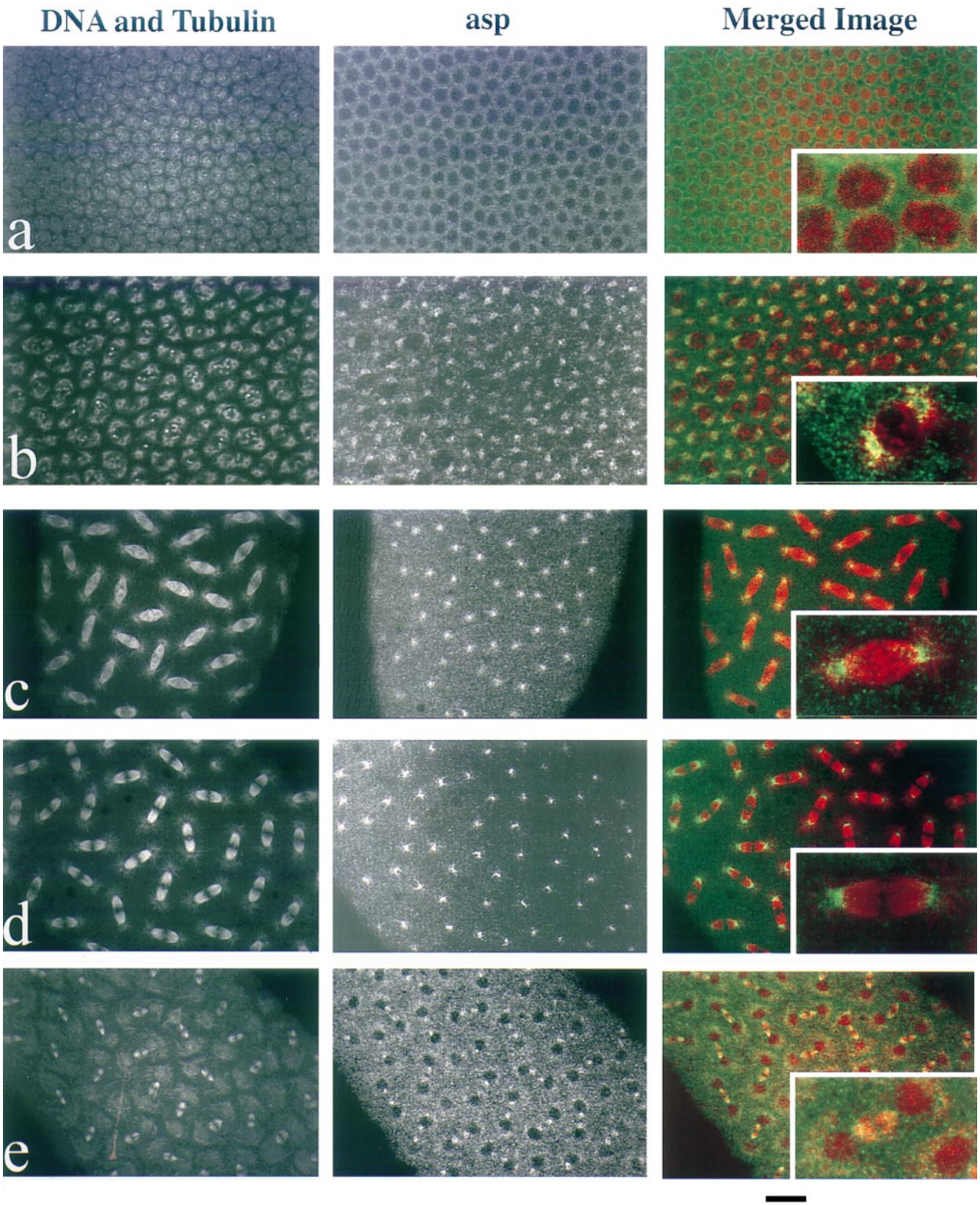

Figure 8. Immunolocalization of Asp in the mitotic cycle in syncytial embryos. Simultaneous staining for DNA with propidium iodide and tubulin with YL1/2 primary antibody and rhodamine-conjugated goat anti–rat IgG are shown in the first column and subsequent red channel of the merged image. Staining 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, 1986*a*,*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^{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 (SPB) 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 *KAR1* 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^{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 centrosomes 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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