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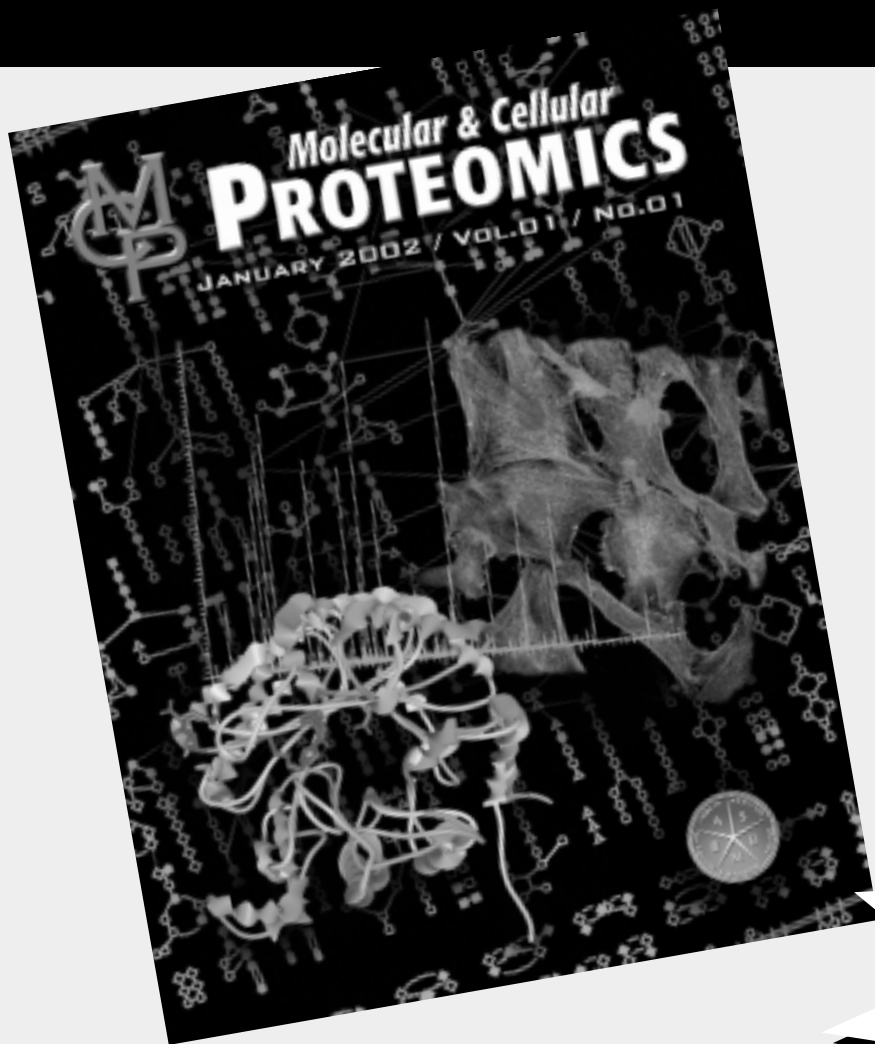
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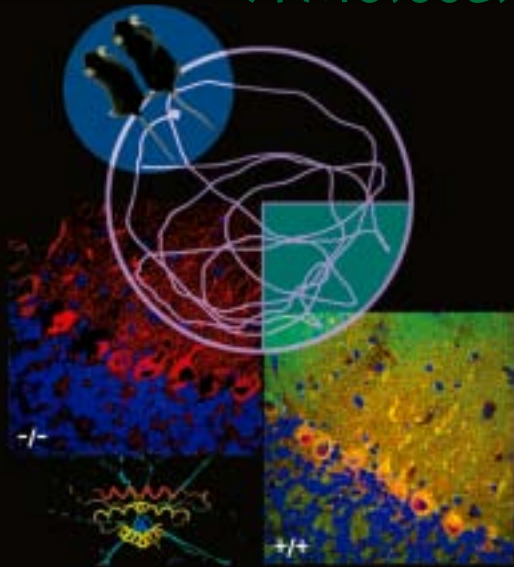
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Two Protein 4.1 Domains Essential for Mitotic Spindle and Aster Microtubule Dynamics and Organization *in Vitro**

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AQ: A

Multifunctional structural proteins belonging to the 4.1 family are components of nuclei, spindles, and centrosomes in vertebrate cells. Here we report that 4.1 is critical for spindle assembly and the formation of centrosome-nucleated and motor-dependent self-organized microtubule asters in metaphase-arrested *Xenopus* egg extracts. Immunodepletion of 4.1 disrupted microtubule arrays and mislocalized the spindle pole protein NuMA. Remarkably, assembly was completely rescued by supplementation with a recombinant 4.1R isoform. We identified two 4.1 domains critical for its function in microtubule polymerization and organization utilizing dominant negative peptides. The 4.1 spectrin-actin binding domain or NuMA binding C-terminal domain peptides caused morphologically disorganized structures. Control peptides with low homology or variant spectrin-actin binding domain peptides that were incapable of binding actin had no deleterious effects. Unexpectedly, the addition of C-terminal domain peptides with reduced NuMA binding caused severe microtubule destabilization in extracts, dramatically inhibiting aster and spindle assembly and also depolymerizing preformed structures. However, the mutant C-terminal peptides did not directly inhibit or destabilize microtubule polymerization from pure tubulin in a microtubule pelleting assay. Our data showing that 4.1 is a crucial factor for assembly and maintenance of mitotic spindles and self-organized and centrosome-nucleated microtubule asters indicates that 4.1 is involved in regulating both microtubule dynamics and organization. These investigations underscore an important functional context for protein 4.1 in microtubule morphogenesis and highlight a previously unappreciated role for 4.1 in cell division.

AQ: B

Protein 4.1, formerly characterized solely as a crucial membrane skeletal protein in mature red cells, is now also recognized to be an important multifunctional structural protein family in nucleated cells. Although protein 4.1 can be plasma membrane-associated in nucleated cells, it also is detected at diverse and interesting subcellular locations during the cell cycle. Protein 4.1 isoforms localize within the nucleus and at centrosomes during interphase, at spindle poles during mitosis,

* This work was supported by Grants DK59079 (to S. W. K.), GM057839 (to R. H.), and DK32094 (to S. W. K., J. A. C., and N. M.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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in perichromatin at anaphase, and in the midbody at telophase (1–3). Whereas mature red cells express only 80-kDa 4.1, the complex subcellular localization patterns of 4.1 in mammalian cells likely results from expression of several 4.1 isoforms, post-translational modifications and expression of multiple 4.1-related genes (4.1R, G, B, and N) (4–14).

Beyond the characterization of its localization, the current challenge is to decipher functions of 4.1 in various subcellular structures. Although the list remains incomplete, a number of protein 4.1 binding partners have been identified to interact with a specific 4.1 domain in red cells and/or nucleated cells, providing some clues as to potential 4.1 functions (see Fig. 1). Prototypical protein 4.1 (R, red cell) contains several functional domains. An N-terminal extension present only in some isoforms in nucleated cells has been found to interact with the centrosomal protein CPAP (centrosome protein-4.1 associated protein) (15). The FERM (4.1/erzin, radixin/moesin) domain interacts with plasma membrane-binding proteins and was recently discovered to also contain a microtubule binding site (16). The spectrin-actin binding domain (SABD¹) is capable of forming ternary complexes with spectrin and actin. The C-terminal domain is of particular interest, because it has been found to interact with NuMA (nuclear mitotic apparatus protein) (17). These observations suggest that 4.1 plays diverse roles within the cytoskeleton.

Fn1

Recently we showed (18, 19) that 4.1 is essential for the assembly of functional nuclei in interphasic *Xenopus* egg extracts and requires its capacity to bind actin, which is found closely associated with 4.1 on nuclear filaments in mammalian cells. This latter observation was recently confirmed and extended in a study (20) that characterized an extensive system of nuclear pore-linked filaments in *Xenopus* oocytes that contain actin and 4.1 epitopes. Here we provide evidence that 4.1 is also essential during mitosis in *Xenopus* egg extracts for proper polymerization and organization of the microtubule cytoskeleton.

During interphase, centrosomes nucleate and organize a radial array of microtubules. We characterized protein 4.1 previously as an integral centrosome component, resisting vigorous salt/detergent extraction and present in centrosomes independent of microtubules. By immunofluorescence and cell whole mount electron microscopy 4.1 epitopes localized on centrioles, in the pericentriolar matrix, and on the fibers connecting the centriolar pair (1). During mitosis duplicated centrosomes become the poles of the mitotic spindle apparatus, contributing to the organization of microtubules into a bipolar array with their minus ends focused at the poles and their plus ends interacting with chromosomes and overlapping within the center of the

AQ: C
AQ: D

¹ The abbreviations used are: SABD, spectrin-actin binding domain; NuMA, nuclear mitotic apparatus protein; PIPES, 1,4-piperazinediethanesulfonic acid; R, red cell.

spindle. Ultimately responsible for accurate chromosome segregation, spindle function is also dependent on a variety of microtubule-based motor proteins including dynein and kinesin-related proteins that cross-link and sort microtubules according to their structural polarity, and mediate chromosome interactions within the spindle. Proper spindle pole organization is known to depend on the function of NuMA, which interacts with dynein and contributes to microtubule minus-end cross-linking to maintain spindle pole structure (as reviewed in Ref. 21) (22–26). Taken together, the observations that protein 4.1 binds to NuMA and localizes to centrosomes and mitotic spindle poles raise the intriguing hypothesis that protein 4.1 is involved in cell division.

In this report we used *Xenopus* egg extracts, a powerful system for *in vitro* dissection of mitotic microtubule assembly, organization, and function. *Xenopus* eggs are laid arrested in the metaphase of meiosis II by cytostatic factor, until fertilization triggers a calcium wave promoting entry into the first mitotic cell cycle. Extracts prepared from unfertilized eggs maintain the cytostatic factor metaphase arrest. Mitotic microtubule asters analogous to spindle poles can be assembled in cytostatic factor extracts through the addition of purified centrosomes that directly nucleate microtubules, or by the addition of microtubule stabilizing agents such as Me_2SO or taxol that induce microtubule polymerization. In the absence of centrosomes, asters are progressively organized into a focused array of minus ends by motor proteins including dynein and are similarly dependent on the activity of NuMA (24, 27). Complete mitotic spindle assembly can be induced by adding demembrated *Xenopus* sperm nuclei, as the centriole-containing basal body of the flagellum remains tightly attached to the sperm and becomes competent to nucleate microtubules in the extract, defining the spindle poles. Thus, the *Xenopus* system can be used to probe both centrosome-nucleated and motor-dependent organization of microtubules into asters, as well as the more complex events of mitotic spindle assembly.

Taking advantage of the open nature of this cell-free system, we present data establishing that protein 4.1 is essential for assembly of these microtubule-based structures by demonstrating that 4.1 depletion of extracts produces aberrant structures. Proper assembly can be restored by the addition of recombinant 4.1. Furthermore we have identified two 4.1 domains critical for spindle, centrosome, and microtubule aster assembly in assays utilizing dominant negative peptides corresponding to 4.1 domains. Our results indicate that 4.1 is involved in regulating both microtubule dynamics and organization and underscore an important functional context for protein 4.1 in cell division.

EXPERIMENTAL PROCEDURES

Materials—Expression vectors for His₆ fusion peptides were either pMW172 (the gift of Dr. M. Way, European Molecular Biology Laboratory, Heidelberg, Germany) or pET 28 (Novagen). The antibody against NuMA was a very generous gift of Dr. A. Merdes (U. Edinburgh, Scotland). IgGs against 4.1R SABD and 4.1R C-terminal domain were described (2). Fluorescent secondary antibodies were from Molecular Probes. Bovine brain tubulin was prepared according to Ashford *et al.* (28).

***Xenopus* Extracts and Assembly Reactions**—10,000 × *g* cytoplasmic *Xenopus* egg extracts and demembrated sperm nuclei were prepared as described (29). For spindle assembly, demembrated *Xenopus* sperm were added to 20 μl of egg extract on ice with 0.2 mg/ml Texas Red-labeled tubulin, reactions incubated at 20 °C for 30–45 min., diluted with BRB80 (80 mM PIPES, 2 mM MgCl₂, 1 mM EGTA, pH 6.8) containing 30% glycerol and 1% Triton X-100 and spun through BRB80 cushions with 40% glycerol onto coverslips (30). Self-organized microtubule asters were assembled by addition of Me_2SO (final concentration 5%) to the egg extract and incubation for 15 min at 20 °C (31). Centrosome asters were assembled for 15 min at 20 °C after the addition of 1 μl of KE37 centrosomes at 2 × 10⁸/ml, prepared according to Moudjou and Bornens (32), to 20 μl of extract that had been centrifuged for 30 min at 60,000 rpm in a TLA 100.3 rotor. Centrosome or self-organized

aster reactions were diluted with BRB80 containing 15% glycerol and 1% Triton X-100 and spun onto coverslips through a cushion of BRB80 containing 30% glycerol.

Indirect Immunofluorescence—*In vitro* assembled structures on coverslips were fixed in −20 °C MeOH and probed by immunofluorescence as described (33). The concentrations of primary antibodies were: SABD IgG, 5 μg/ml; C-terminal domain IgG, 10 μg/ml; anti-NuMA, 1:50 dilution. Secondary antibodies were used at a 1:100 dilution. Samples probed with equal amounts of control non-immune IgG or without primary antibody or sera showed no fluorescent patterns. Images were captured using a Nikon Eclipse 2000 microscope equipped with a CCD camera and processed using Adobe PhotoShop. Under the imaging conditions used, the limits of resolution of overlap between two fluorophores (*e.g.* superimposition of red and green signals to generate yellow coloration) was estimated to be ~300 nm.

Expression and Purification of His₆-tagged Proteins—Protein 4.1-related peptides were expressed and purified as described (19). Assays with His₆ peptides (1–8 μg) were preincubated on ice for 10 min then incubated at 20 °C for assembly of spindles, centrosome asters, or self-organized asters as indicated. Although a range of 1–8 μg was tested for each peptide, the data presented are from experiments using 8 μg of the indicated peptide.

Microtubule Co-pelleting Assay—Solutions of 4.1 peptides and of 35 μM tubulin and 1 mM GTP in BRB80 were precleared by centrifugation in a TL-100 rotor for 15 min at 40,000 rpm at 4 °C. Reactions containing 1 mM GTP, 35 μM tubulin, 35 μM peptide were mixed on ice, Me_2SO was added (final concentration 5%), the reaction was incubated for 30 min at 37 °C, layered onto 40%/BRB80 sucrose cushions, and microtubules were pelleted at 40,000 rpm for 20 min in a TL-100 rotor. Equivalent amounts of supernatant and pellet were analyzed by Western blotting of a 15% SDS-polyacrylamide gel. By this assay, inhibitors of microtubule formation show tubulin remaining in the supernatant (34).

Immunodepletion and Rescue—For 4.1 depletion from *Xenopus* extracts, protein G-coupled magnetic beads (Dynal) from 100 μl of slurry were mixed with 15 μg of 4.1R SABD, C-terminal domain IgGs or non-immune rabbit IgG for 1 h at 4 °C, the beads were washed twice with 0.1 M sodium phosphate buffer, pH 7.0 (57.7% Na₂HPO₄ and 42.3% NaH₂PO₄, v/v), and three times with XB buffer (29) then divided into three aliquots. Extract (100 μl) was successively depleted three times by rotation with IgG-coupled beads at 4 °C for 1 h, beads were collected magnetically, and extract was used for the assembly of spindles, centrosome asters, or self-organized asters. Extract depletion was estimated by densitometry of Western blots using an Alpha Imager 2200 and software. In rescue experiments, 1–9 μg of purified bacterially expressed 80-kDa 4.1R was added to 20-μl reactions and incubated on ice for 10 min prior to the initiation of assembly. Reactions in three independent experiments were sampled during 15–45-min incubation periods for the assembly of spindles, centrosomes, or asters. The experiment presented was performed in parallel using the same depleted extract as described (19).

RESULTS

4.1 Localizes to Mitotic Spindles, Centrosome Asters, and Self-organized Asters Reconstituted in *Xenopus* Egg Extracts—Previous reports (1, 2, 16, 17) using a variety of mammalian cells established that 4.1 is localized to centrosomes and mitotic spindle poles. As the *Xenopus laevis* 4.1 sequence has many highly homologous regions relative to mammalian family members, including the SABD and C-terminal domains (Fig. 1) (19), we anticipated a similar 4.1 localization in *Xenopus*. Furthermore, 4.1 function was shown to be conserved between the frog and mammals in studies reconstituting properties of 4.1-deficient human erythrocyte membranes using a recombinant *Xenopus* 4.1 domain (35). We first verified that 4.1 epitopes could be detected in centrosomes and mitotic spindles of cultured *Xenopus* fibroblasts (data not shown). Next we incubated cytostatic factor-arrested *Xenopus* egg extracts either with demembrated *Xenopus* sperm to assemble mitotic spindles or with centrosomes from KE37 cells to reconstitute centrosome-based microtubule arrays (referred to as centrosome asters). We also induced the motor-dependent assembly of stabilized microtubules into asters by adding 5% Me_2SO to the egg extract (referred to as self-organized asters). This allowed us to examine both centrosome-nucleated and motor-organized structures. In

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Protein 4.1 Domains in Spindle and Centrosome Assembly

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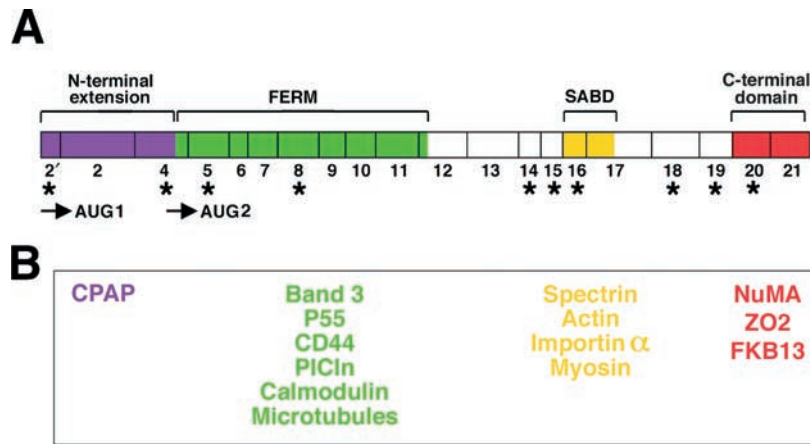


FIG. 1. Domain organization of protein 4.1 and interacting proteins. *A*, a schematic map of 4.1R indicating functional interacting domains. Exon numbers appear below the bar with asterisks indicating alternatively spliced exons. Arrows indicate translation initiation sites. Isoforms initiated at AUG1 include the N-terminal extension (exons 2–4). The membrane binding 30-kDa/FERM domain extends from exon 4 to 12. In this report, the spectrin-actin binding domain (SABD) refers to amino acid sequences from exons 16 and partial 17, whereas the C-terminal domain denotes peptides from exons 20 to 21. *B*, proteins known to interact with protein 4.1 domains. The proteins are listed under and color-coded corresponding to their 4.1 interaction domain. CPAP refers to centrosome protein-4.1 associated protein (15).

each reaction, microtubules were stained by the addition of trace amounts of rhodamine-labeled tubulin. When each of these structures was probed by immunofluorescence microscopy using affinity-purified antibodies against the 4.1R SABD or C-terminal domain or against the 80-kDa 4.1R, strong 4.1 signals were concentrated at the minus ends of microtubules focused at the spindle poles and also in the center of both centrosome and self-assembled asters (Fig. 2).

Immunodepletion of 4.1 Compromises Assembly of Microtubule-based Structures and Can Be Rescued by Supplementation with Recombinant 4.1R—To test whether the protein 4.1 itself is essential for the assembly of spindles, centrosome asters, and self-organized asters, we depleted 4.1 from *Xenopus* extracts using 4.1 domain-specific affinity-purified IgGs bound to protein G magnetic beads. Previously (19) we showed by Western blotting that *Xenopus* extracts contain protein bands from ~47 to 110 kDa detected by SABD and C-terminal domain IgGs, which were reduced by 50–100% after three rounds of antibody depletion. Using 4.1-immunodepleted extracts, we evaluated the morphology of spindles, centrosomes, and self-organized microtubule asters, and as an additional indicator of structural integrity we localized the 4.1 binding partner/spindle pole protein NuMA by immunofluorescence. In controls, NuMA largely localized with 4.1 epitopes in a tight focus at the spindle poles, and in the centers of centrosome and self-organized microtubule asters (Figs. 2 and 3).

Normal spindles, centrosome asters, and self-organized asters assembled in egg extracts mock-depleted with nonspecific IgG and protein G beads (Fig. 3A). In contrast, there was a dramatic morphological disruption of all microtubule structures assembled in extracts depleted with either 4.1 domain-specific SABD or C-terminal domain IgGs. In spindles assembled in either depleted extract, chromosomes were not aligned equidistant from the poles but were looped out of the spindle midzone (Fig. 3A). Spindles formed in SABD-depleted extracts most often were multipolar, whereas those from C-terminal domain-depleted extracts generally had large unfocused poles. Centrosome asters were disorganized microtubule arrays without an obvious focal center revealed by NuMA staining (Fig. 3B). In SABD-depleted extracts, centrosomes asters often contained multiple small NuMA foci radiating several bundles of microtubules. Similarly, self-organized asters assembled in either the C-terminal domain or SABD-depleted extracts had disorganized microtubules, and NuMA was mislocalized (Fig. 3C). Therefore, depleting SABD- and C-terminal domain-con-

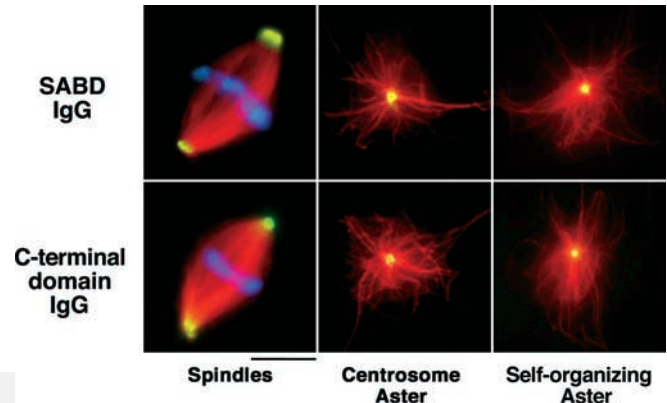


FIG. 2. Protein 4.1 epitopes detected by immunofluorescent microscopy in spindles, centrosome asters, and self-organized microtubule asters assembled *in vitro* in *Xenopus* egg extracts. Localization of DNA in spindles was detected by 4,6-diamidino-2-phenylindole (blue), and microtubules were labeled with rhodamine tubulin (red). Protein 4.1 epitopes were visualized by indirect immunofluorescence using affinity-purified IgG directed against the 4.1 SABD and C-terminal domain as described previously (2). Both IgGs produced protein 4.1 signals (green) at spindle poles and at the minus ends of microtubules oriented toward the center of centrosome asters and self-organized asters. The yellow coloration in these merged images indicates apparent colocalization of 4.1 epitopes and microtubules. Bar = 20 μ m

taining 4.1 proteins disrupted spindle, centrosome aster, and self-organized microtubule aster assembly.

The aberrant reconstitution of microtubule structures observed in depleted extracts could result either from loss of 4.1 function itself or from loss of an essential 4.1 protein binding partner co-depleted in the reaction. To address this issue we added back-purified recombinant 80-kDa 4.1R to depleted extracts. Strikingly, spindle, centrosome, and aster reconstitution was completely restored by supplementation with recombinant 4.1R in extracts depleted with either SABD or C-terminal domain IgGs, producing structures with morphology and NuMA distribution comparable with controls (Fig. 3, A', B', and C'). The rescue of assembly by recombinant 4.1R shows directly that protein 4.1 is essential for the assembly of mitotic spindles, centrosome, and self-organized microtubule asters. Furthermore, this result indicates that a single isoform containing both SABD and C-terminal domains is sufficient to mediate all of the functions of 4.1 necessary for its role in organizing mitotic microtubule arrays in *Xenopus* egg extracts.

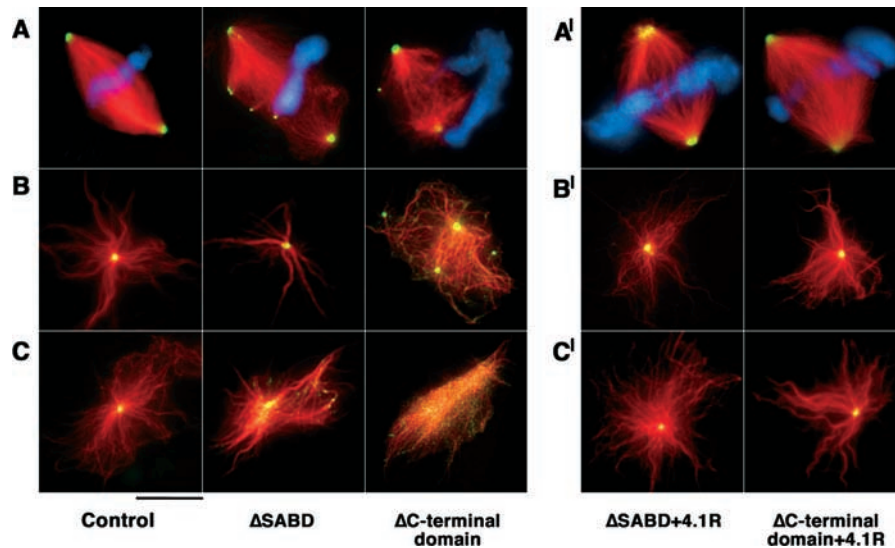


FIG. 3. Aberrant spindle, centrosome aster, and self-organized aster assembly *in vitro* in 4.1-depleted extracts and rescue of defective phenotype by supplementation with recombinant 80-kDa 4.1. In the merged images, DNA in spindles was detected with 4,6-diamidino-2-phenylindole (blue), microtubules are red, and NuMA is green. Yellow indicates an overlap of red and green signals. *A*, the products of spindle assembly reactions in *Xenopus* extracts depleted using SABD IgG (Δ SABD) or C-terminal domain IgG (Δ C-terminal domain) were severely perturbed relative to spindles assembled in control extracts (IgG). Bar, 20 μ m. *B*, centrosome asters assembled in *Xenopus* extracts depleted using SABD IgG or C-terminal domain IgG were aberrant both with respect to microtubules and NuMA distribution relative to centrosome asters assembled in control extracts. *C*, self-organized microtubule asters formed after Me_2SO addition to extracts depleted using SABD IgG or C-terminal domain IgG were disoriented and had a scattered distribution of NuMA epitopes. *A'*, *B'*, *C'*, spindles, centrosome asters, and self-organized asters assembled after the addition of recombinant 80-kDa 4.1 (+4.1) to depleted extracts. With the addition of 9 μ g of 80-kDa 4.1 spindles, centrosome asters, and self-organized asters had similar morphology, size, and distribution of NuMA as to their respective controls.

SABD related peptides

4.1N 16-17 -----KPEQETTPRHK--QEFLLDKPEDVLLKHOASINELKRTLKPEPNSKLIHFRDRDWRERRRLPS
 4.1R 16-17 KRERLDGENIYIRHSNLMLEDLDKSEQEIKKHASISELKKNFME--SVPEPRPSEWDKRLSTHT
 4.1R 16-17 Δ NF KKRERLDGENIYIRHSNLMLEDLDKSEQEIKKHASISELKK**ME--SVPEPRPSEWDKRLSTHT

C-terminal domain related peptides

4.1R 20-21 TVKGGISETRIEKRIVITGDADIDHDQVLVQAIKEAKEQHPDMSVTKVVVHQETEIA
 4.1R 20-21 mut3V TVKGGISETRIEKRIVITGDADIDHDQVLVQAIKEAKEQHPDMSATKAVAHQETEIA

FIG. 4. Amino acid sequences of expressed peptides related to 4.1 SABD and C-terminal domain added to *in vitro* spindle, centrosome-nucleated, and self-organized aster assembly reactions. The 4.1R SABD peptide corresponds to a region encoded by exons 16–17. The 4.1N 16–17 peptide corresponds to the 4.1R SABD region but with low homology except for the boxed amino acids. The 4.1R 16–17 Δ NF has amino acids identical to 4.1R 16–17 (wild type 4.1R SABD) except for deletions of asparagine and phenylalanine in exon 17 (indicated by asterisks). The 4.1R C-terminal domain peptide corresponds to a region encoded by exons 20–21, and the 4.1R 20–21 mut3V peptide has an identical sequence except that three valines were mutated to alanines (underlined) This figure is modified from Krauss *et al.* (19).

Dominant Negative 4.1 Peptides Distort Assembly of Spindles, Centrosomes, and Microtubule Asters in Vitro—Having established that 4.1 is required for proper formation of microtubule structures, we next wanted to test the functions of specific 4.1 domains in spindle, centrosome, and microtubule aster assembly. To this end we added to *in vitro* assembly reactions bacterially expressed peptides with amino acid sequences corresponding to 4.1 domains. We reasoned that the peptides might act competitively to disrupt 4.1 complexes or to sequester important 4.1 binding partners during the assembly process. We analyzed peptide effects both on morphology and localization of the 4.1 binding partner NuMA as another measure of functional disruption because in controls NuMA localized in a tight focus at spindle poles, in the pericentriolar area of centrosomes and at the centers of self-organized asters (Fig. 3).

Initially we analyzed the effects of peptides related to 4.1 SABD and C-terminal domains. We focused on these domains because they (a) have important defined functions, (b) are highly conserved between frog and mammals, (c) were present in recombinant 4.1R used to rescue extracts immunodepleted by SABD and C-terminal domain IgGs, and (d) were demonstrated to profoundly distort nuclear assembly *in vitro* in egg extracts (19). For our experiments, we expressed His₆-tagged

peptides encoded by either exons 16–17 (amino acids 644–705) in the 4.1R spectrin-actin binding domain (SABD) or by exons 20–21 (amino acids 800–858) of the 4.1R C-terminal domain (Fig. 4). As controls for the SABD peptide, we used a variant 4.1N SABD peptide with low amino acid sequence homology to the 4.1R 16–17 peptide and a 4.1R 16–17 Δ NF peptide with a deletion of two amino acids within its actin binding domain rendering it unable to bind actin but retaining spectrin binding. As a control for the 4.1R C-terminal domain peptide we used a C-terminal domain peptide in which three valines were changed to alanines, an alteration previously shown to reduce its binding affinity to NuMA Tail I fragment by ~60-fold (19).

Addition of the 4.1R 16–17 SABD peptide produced spindles with less focused microtubules at the poles and more dispersed NuMA in the peripolar area. Often there were multipolar structures. In these spindles, chromatin appeared less condensed at the metaphase plate (Fig. 5A, top row). Centrosome asters, while retaining a small central focus of NuMA staining, had only a few bundled microtubules radiating outward (Fig. 5A, middle row). Self-organized asters also appeared to contain bundled microtubules and had a very diffuse central area of NuMA with additional NuMA epitopes distributed along microtubule bundles (Fig. 5A, bottom row). However, an equal

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Protein 4.1 Domains in Spindle and Centrosome Assembly

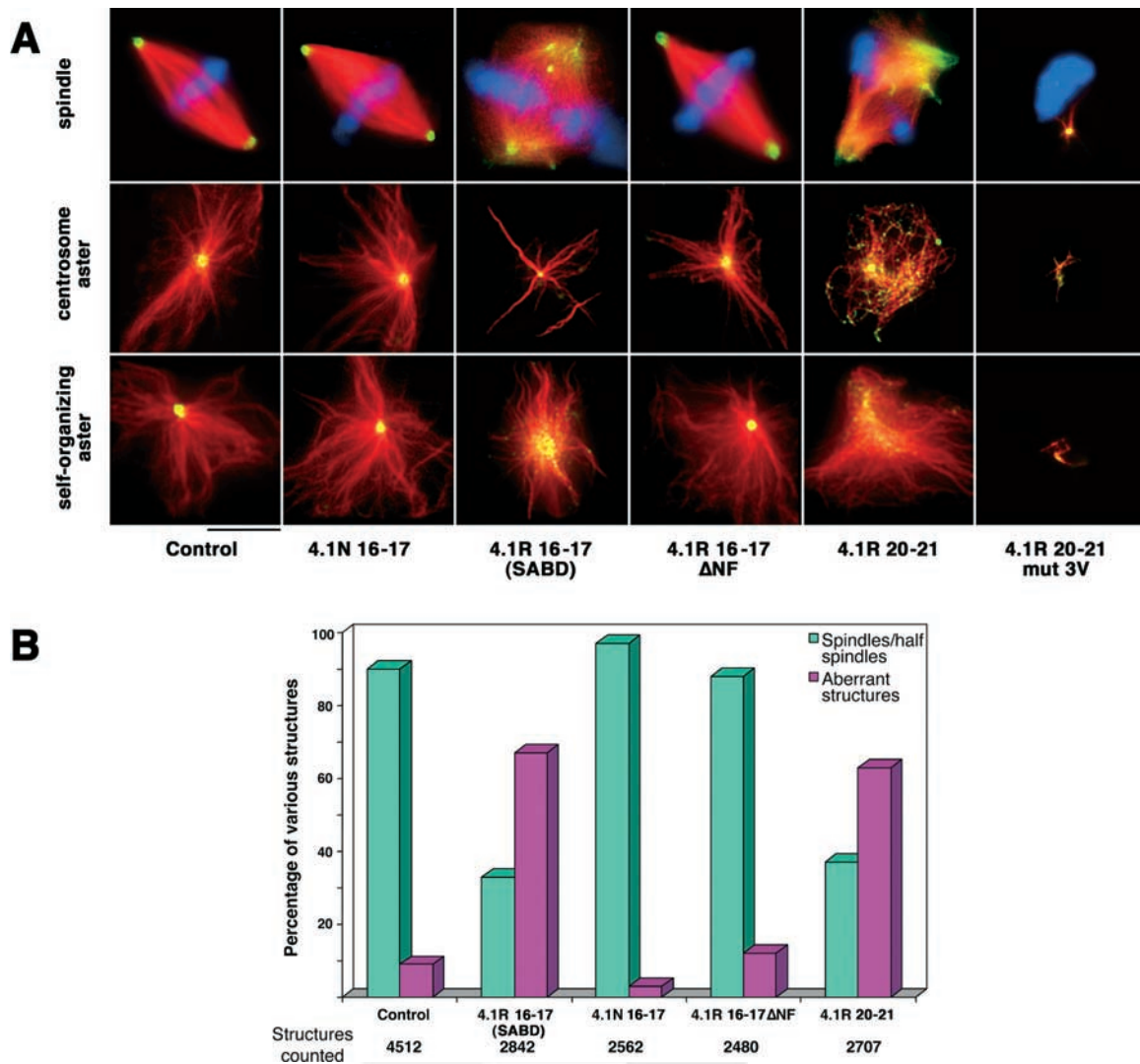


FIG. 5. Effects of 4.1 peptides on assembly of spindles, centrosome asters, and self-organized microtubule asters in *Xenopus* mitotic egg extracts *in vitro*. *A*, the products of assembly reactions with the indicated His₆ peptides were visualized by immunofluorescence. Reactions were spiked with fluorescent tubulin (*top*, red) and probed with antibodies against NuMA (*green*). DNA (*blue*) was stained by 4,6-diamidino-2-phenylindole. Neither variant 4.1N 16–17 nor 4.1R 16–17ΔNF control peptides markedly affected the ordered assembly of structures, whereas both 4.1R 16–17 SABD and 4.1R C-terminal domain peptides (4.1R 20–21) had profound effects on the organization of microtubules and NuMA. The addition of 4.1R 20–21 mut3V peptides resulted in decondensed DNA and extremely truncated microtubules. All images within horizontal register are at equal magnification. *Bar*, 20 μm. *B*, quantitation of spindle assembly perturbation by 4.1R 16–17 SABD and 4.1R C-terminal domain peptides. Control reactions included buffer alone, variant 4.1N 16–17 peptides, or 4.1R 16–17ΔNF peptides. Total number of structures counted is indicated *below the bars*. Data from reactions containing 4.1R 20–21 mut3V peptides are not presented because perturbation was so profound that even surviving “structures” were abnormal relative to controls.

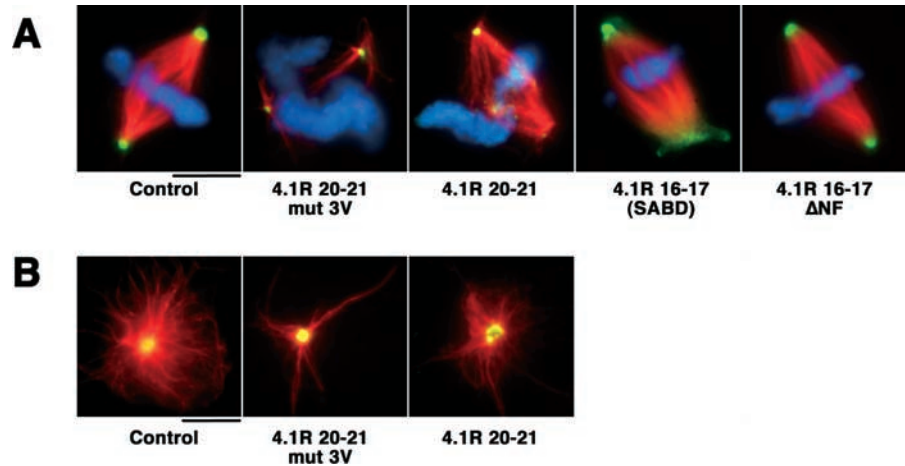
concentration of a deletion mutant in the 4.1R SABD peptide (4.1R 16–17ΔNF) added to extracts did not affect the assembly of spindles, centrosomes, or asters with respect to the morphology or distribution of NuMA (Fig. 5A). A variant 4.1N SABD peptide also did not cause any apparent perturbation (Fig. 5A). Therefore the dominant negative effects of the 4.1R SABD peptide on proper structural assembly are sequence-specific and require its capacity to bind actin.

The C-terminal domain was also critical for the assembly of normal spindles, centrosomes, and microtubule asters. C-terminal domain peptide addition resulted in structures even more distorted than those treated with SABD peptides. Spindles had unfocused poles or were multipolar and were also bent in most cases. Centrosome-nucleated and self-organized asters were not radiating from a discernable center, but appeared as microtubule “mats” with irregularly distributed NuMA (Fig. 5A). Although the importance of NuMA in proper spindle formation is well documented, these observations imply that the interaction of NuMA and 4.1 is also crucial for centrosome and

microtubule aster formation *in vitro*. To test this, the mutant C-terminal domain peptide with decreased NuMA affinity was added to the assembly reactions. Surprisingly this peptide had dramatic and unpredicted effects. In both centrosome and aster assembly reactions, no microtubules of normal length were observed but only small NuMA foci were detected with extremely short and sparse microtubules (Fig. 5A). In spindle reactions, *Xenopus* sperm DNA was decondensed and had juxtaposed a single polar remnant. Quantification of these effects shows that dominant negative effects of 4.1R SABD and C-terminal domain peptides on microtubule-based structures are profound and sequence-specific (Fig. 5B).

Protein 4.1 Function Is Required Continuously to Maintain Proper Microtubule Organization in Mitotic Structures—Our results indicate that 4.1 is essential for generating properly polymerized and organized microtubule asters and spindles. We wanted to test whether 4.1 function becomes dispensable once structures have assembled. This question could easily be addressed in *Xenopus* egg extracts, because inhibitory peptides

FIG. 6. Effects of 4.1 peptides on structural maintenance of mature spindles and self-organized microtubule asters. *A*, after assembly of spindles in extracts 4.1 peptides were added and incubated for an additional 15 min. Structures were spun onto coverslips and analyzed by immunofluorescence. In the merged images, DNA was stained by 4,6-diamidino-2-phenylindole (blue), microtubules are red, and NuMA is green. Bar, 20 μ m. *B*, microtubule asters formed after an addition of 5% Me₂SO to extracts were further incubated for 15 min with 4.1 peptides, and structures were spun onto coverslips. Coincidence of red (microtubules) and green (NuMA) signals generates a yellow coloration. Bar, 15 μ m.



could be added at any time to assembly reactions.

We tested whether microtubules in bipolar spindles and self-organized asters could survive exposure to C-terminal domain mut3V peptides by adding C-terminal domain mut3V peptides to extracts containing normal preformed structures. After a 15-min incubation, few microtubules remained in spindles. These microtubules appeared to extend from pole remnants demarcated by very small NuMA-staining foci and were markedly “slackened.” Chromatin was decondensed and not positioned at a spindle equator, extending beyond the microtubules (Fig. 6A). Incubation with wild type C-terminal domain peptide also compromised structure but much less severely than C-terminal domain mut3V mutant peptides. Spindle microtubules became less rigidly and symmetrically organized with smaller less focused NuMA staining in the pole areas. Some NuMA was also unevenly distributed on chromatin (Fig. 6A). Incubation with SABD peptides dispersed peripolar NuMA, whereas SABDΔNF peptides did not appear to compromise spindle morphology or NuMA localization.

To test more stringently the effects of 4.1 C-terminal domain peptides on microtubules arrayed independently of centrosomes, self-organized asters stabilized by Me₂SO were incubated with 4.1 peptides. After the exposure of microtubule asters to C-terminal domain mut3V peptides, there remained on average 3–8 microtubule bundles emanating from a NuMA-containing area similar in size to controls (Fig. 6B). Incubation with wild type C-terminal domain peptides produced a different structure phenotype having markedly truncated microtubules symmetrically arrayed around a NuMA region less tightly focused than in controls. In some orientations, a central hollow area or ring-like distribution of NuMA was detected (Fig. 6B). Because most mature microtubules in spindles as well as self-organized asters did not survive exposure to either mutant or wild type C-terminal domain peptides, it appears that continuous 4.1 function is required to maintain proper microtubule polymerization and organization.

The deleterious effects on mature spindles and asters could result from direct or indirect microtubule destabilization mechanisms. To investigate whether 4.1 peptides directly bind or destabilize microtubules, we performed a sedimentation assay using purified tubulin polymerized *in vitro* that compares the polymerized tubulin polymer (in the pellet) with unpolymerized tubulin (in the supernatant fraction). In control reactions without tubulin, none of the 4.1-related peptides pelleted under microtubule polymerizing conditions (Fig. 7, –*tub* lanes). When added to microtubule polymerization reactions, SABD peptides remained entirely in the supernatant and did not appreciably alter the amount of microtubules pelleted. In reactions with either C-terminal domain or C-terminal domain mut3V peptides, each

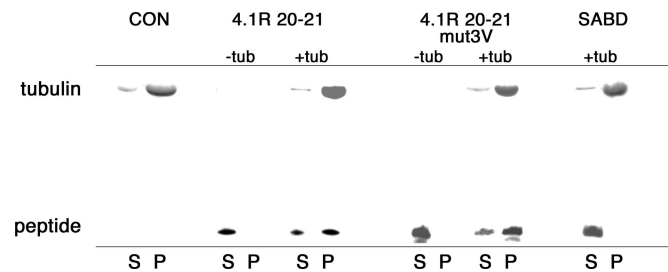


FIG. 7. Analysis of *in vitro* association of 4.1 peptides and microtubules. Western blots of equivalent aliquots of pellets (*P*) and supernatants (*S*) from co-pelleting assays were probed with antibodies against tubulin and His₆. In all tubes containing tubulin (+*tub*), microtubules readily formed as detected in pellet fractions regardless of the presence of various peptides. When tubulin was omitted, the peptides remained entirely in the supernatant. However, in reactions with tubulin and C-terminal domain mut3V peptides (*4.1R 20–21 mut3V*) or C-terminal domain (*4.1R 20–21*) but not SABD peptides, a fraction of the peptides was detected in microtubule pellets.

peptide was detected both in the microtubule pellet and in the supernatant (Fig. 7, +*tub* lanes). However, the amount of tubulin in the supernatant and pellet was similar to controls or when SABD peptide was added. Therefore, whereas both C-terminal domain-related peptides showed some binding to microtubules and SABD peptides did not, the 4.1 peptides did not have measurable direct microtubule destabilizing activity.

DISCUSSION

Protein 4.1 Is Essential for Spindle and Centrosome Assembly—Protein 4.1 interactions in mammalian red cells serve to link and stabilize structural components in the membrane skeleton with integral membrane proteins. Protein 4.1 is also widely distributed in many tissues and localizes at several distinct subcellular sites crucial for cell division in nucleated cells. By analogy to its critical role in red cell membrane mechanics, protein 4.1 may link or stabilize components in nuclei, centrosomes, and mitotic spindles, providing both structural organization and flexibility necessary for dynamic cytoskeletal assembly and disassembly during the cell cycle. To begin to address 4.1 function in microtubule morphogenesis, we exploited the power of *in vitro* reconstitution using *Xenopus* egg extracts after first establishing 4.1 localization at *in vitro* assembled spindles, centrosomes, and self-organized microtubule asters. In depletion/add-back experiments, markedly aberrant structures formed in depleted egg extracts, but the addition of purified recombinant 80-kDa 4.1R protein restored normal centrosome and spindle assembly demonstrating that 4.1 is essential. Because 4.1 was required for arraying microtubule asters by motor-driven self-organization as well as by microtubule

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nucleating centrosomes, the requirement for 4.1 to properly organize and orient microtubules is not dependent exclusively on centrioles.

Although rescue was with recombinant 80-kDa 4.1R containing both a SABD and C-terminal domain, it is possible that 4.1 rescue may not be exclusive to that isoform or even to the 4.1R family. Multiple 4.1R splice variants exist, and recent transfection studies (16) showed that apparent co-localization of 4.1R with microtubules or disruption of microtubule organization may be isoform- and tissue-specific. Furthermore, another generally expressed 4.1 family member (4.1G) also contains an SABD region that can form a ternary complex with spectrin and actin (36) and a highly homologous 4.1G C-terminal domain that can bind NuMA (37). Because 4.1G colocalizes with NuMA at spindle poles (37) and has been detected at centrosomes,² future experiments will test the rescue of spindle and centrosome assembly in 4.1-depleted extracts using specifically engineered 4.1 recombinant proteins from both the 4.1R and 4.1G families. This approach will facilitate functional mapping of 4.1 sequences required for microtubule organization in addition to the microtubule binding site in the 4.1R FERM (16).

4.1 Peptides From Two Independent Domains Act as Dominant Negatives in Assembly Reactions—To begin to define 4.1 domains critical for aster/spindle assembly and structure we added peptides to egg extracts, initiated assembly and examined morphology of the structures formed. One domain that behaved as a dominant negative was the SABD peptide. Although neither F-actin nor spectrin is reported to be a centrosome or spindle component, an SABD peptide mutant for actin binding did not exert deleterious effects on morphology. This suggests several of the following possibilities. 1) The SABD may be able to interact with contractin/Arp1, a protein >50% homologous to actin required for centrosomal microtubule anchoring and/or focusing (38, 39). Contractin/Arp1 is also a major component of dynactin, an activator of the minus end-directed microtubule motor cytoplasmic (40, 41). This is a particularly attractive hypothesis because disruption of dynein-dynactin complexes or overexpression of contractin/Arp1 α perturbs centrosomes and spindles (25, 42, 43). 2) Interactions between microtubules and actin may contribute to aspects of spindle and centrosome formation. Proteins like MACF have been identified that cross-link these two filaments (44). 3) Non-filamentous actin, present in nuclei (45, 46) could play a role in spindle and centrosome assembly. It was reported that one molecule of actin is present for each dynactin complex (47). Future experiments could test whether actin inhibitors such as latrunculin interfere with spindle and centrosome assembly. 4) The two deleted amino acids in SABD peptides, critical for actin binding (36), may also be part of another interaction site for an unidentified 4.1 binding partner.

The discovery of a NuMA binding site within exons 20–21 of the 4.1R C-terminal domain and that NuMA, dynein, and the dynactin subunit p150^{glued} co-precipitate with 4.1 (17) provided us with insights about possible mechanisms responsible for the dominant negative effects of C-terminal domain peptides on spindle and centrosome assembly. Transport of NuMA toward microtubule minus ends is required for assembly and maintenance of focused spindle poles (25); aster C-terminal domain peptides may disengage NuMA/dynactin as cargo from the dynein motor. This is not unlikely, because the transient nature of the interaction of dynein with dynactin/NuMA has been reported (25, 40). Additionally, NuMA also directly binds to and stabilizes microtubules (48), but stabilization is regulated by other proteins such as LGN (49). Although the neighboring 4.1

binding site on NuMA does not directly overlap the microtubule/LGN binding site of NuMA, 4.1 C-terminal domain peptides may indirectly alter the modulation of microtubule dynamics integral to the assembly of spindle poles and asters. The dense microtubule mats with mislocalized NuMA observed after centrosome or aster assembly with C-terminal domain peptides may reflect NuMA oligomerization and stabilization of microtubules (48, 49).

Protein 4.1 and the Minus Ends of Microtubules—Localization of 4.1 at the minus ends of radially arrayed microtubules in spindles and centrosomes may occur by two mechanisms: 1) transport by minus end-directed motors and/or 2) association with other minus end-associated proteins already on site (reviewed in Ref. 50). At spindle poles/centrosomes 4.1 could function not only as a crucial structural protein but also to regulate interactions within scaffolding complexes such as those containing NuMA (17), γ -tubulin (15),² and pericentrin (1). Some of these interactions appear to be dynamic, because as reported here 4.1 function is required for continuous maintenance of proper mitotic structures. However, many key functions of 4.1 related to microtubules remain to be identified as suggested by another recent study (37) as well as demonstrated by our own data.

In mitotic egg extract assembly reactions, few microtubules survived exposure to C-terminal domain peptides containing changes in only three amino acids critical for NuMA binding. When added to nuclear assembly reactions in interphasic egg extracts, this peptide also produced markedly truncated centrosome microtubule arrays (19). However, C-terminal domain mut3V peptides did not directly destabilize microtubules polymerized from purified tubulin and, in fact, partially co-pelleted with them. This observation indicates the involvement of other extract proteins in co-regulation of microtubule dynamics by 4.1.

Our investigations predict that multiple protein 4.1 domains regulate microtubule dynamics and/or release as well as microtubule organization. Apparently this is accomplished by both direct and indirect mechanisms. Supporting the hypothesis that 4.1 impacts cell division, depletion of 4.1 or addition of 4.1 dominant negative peptides produced multipolar asymmetric spindles and strongly disrupted centrosome organization. Clearly 4.1 misregulation or disruption *in vivo* could lead to spindle and centrosomal aberrations, abnormal chromosome segregation, and cell cycle perturbations, hallmarks of potential pathology.

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