Non-erythroid protein 4.1R (4.1R) consists of a complex family of isoforms. We have shown that 4.1R isoforms localize at the mitotic spindle/spindle poles and associate in a complex with the mitotic-spindle organization proteins Nuclear Mitotic Apparatus protein (NuMA), dynein, and dynactin. We addressed the mitotic function of 4.1R by investigating its association with microtubules, the main component of the mitotic spindles, and its role in mitotic aster assembly in vitro. 4.1R appears to partially co-localize with microtubules throughout the mitotic stages of the cell cycle. In vitro sedimentation assays showed that 4.1R isoforms directly interact with microtubules. Glutathione S-transferase (GST) pull-down assays using GST-4.1R fusions and mitotic cell extracts further showed that the association of 4.1R with tubulin results from both the membrane-binding domain and C-terminal domain of 4.1R. Moreover, 4.1R, but not actin, is a mitotic microtubule-associated protein; 4.1R associates with microtubules in the microtubule pellet of the mitotic asters assembled in mammalian cell-free mitotic extract. The organization of microtubules into asters depends on 4.1R in that immunodepletion of 4.1R from the extract resulted in randomly dispersed microtubules. Furthermore, adding a 135-kDa recombinant 4.1R reconstituted the mitotic asters. Finally, we demonstrated that a mitotic 4.1R isoform appears to form a complex in vitro with tubulin and NuMA in highly synchronized mitotic HeLa extracts. Our results suggest that a 135-kDa non-erythroid 4.1R is important to cell division, because it participates in the formation of mitotic spindles and spindle poles through its interaction with mitotic microtubules.

Protein 4.1R is an 80-kDa structural component of the red blood cell cytoskeleton. 4.1R is critical to the formation of the spectrin/actin/4.1R junctional complex, whose integrity is vital for the mechanical stability and elasticity of the red blood cells (reviewed in Ref. 1). The 80-kDa 4.1R of mature red blood cells is the only type of spectrin isoform in human T cells (1). From these results, we hypothesize that 4.1R plays an important role in organizing mitotic spindle and spindle poles.

A central event during cell division is the transformation of the interphase network of microtubules into a bipolar spindle that mediates the accurate segregation of chromosomes during both mitosis and meiosis. Microtubules in the spindle are organized such that the minus ends are at or near the poles while the plus ends extend toward the cell cortex or chromosomes (2). Recent experiments have begun to define the mechanisms by which microtubules are aligned with the chromosomes during mitosis.

The abbreviations used are: MBD, membrane-binding domain; NuMA, Nuclear Mitotic Apparatus protein; SAB, spectrin/actin binding; CTD, C-terminal domain; HP, head-piece; GST, glutathione S-transferase; DTT, dithiothreitol; Ab, rabbit polyclonal antibodies; mAb, mouse monoclonal antibodies; CHAPS, 3-[3-cholamido(1-propanesulfonic acid].
and molecules involved in organizing microtubules into spindles (reviewed in Ref. 24). In somatic cells, centrosomes are the dominant centers of microtubule nucleation. However, centro- somes and the microtubule arrays nucleated from them are not sufficient to act as functional spindle poles (25). The organization of microtubules into spindles is governed largely by the interaction of microtubules and microtubule ends with accessory proteins that regulate microtubule dynamics. Several non- centrosomal proteins such as cytoplasmic dynein (26), dynactin (27), Eg5 (28), HSET (29), ch-TOP (30), cohesin (31), and astrin (32), as well as the structural protein NuMA (33) are involved in focusing microtubules at spindle poles. Researchers are now actively searching for additional proteins that may associate only transiently with spindle poles and for the proteins that are minor components of the spindle poles.

In this study, we investigate the association of 4.1R with microtubules, the main component of the mitotic spindles, and the role of 4.1R in mitotic aster assembly in vitro. We demonstrate that 4.1R is a mitotic microtubule-associated protein and that 4.1R is critical to the organization of microtubules into asters in HeLa mitotic extracts. Our findings suggest that a non-erythroid 135-kDa 4.1R is an important component of cell division by participating in the formation of mitotic spindles and spindle poles through its interaction with mitotic microtubules.

**EXPERIMENTAL PROCEDURES**

**Antibody Production**—Antisera to synthetic peptides of exon 13 of 4.1R (DSADDRSPRPTSPAPAD (anti-exon 13) and the recombinant HP of 4.1R (anti-HP) were prepared as described previously (20). The sera were purified on immobilized columns using the Sulfolink kit (Pierce) covalently cross-linked to the synthetic peptides or fusion peptides used for immunization. The purified antibodies were eluted from the columns with 0.1 M glycine (pH 2.8), neutralized with Tris-HCl, pH 9.5, and dialyzed into phosphate-buffered saline. Anti-NuMA mAb was purchased from Oncogene. Anti-dynein mAb (intermediate chain, clone 70.1), anti-actin mAb, and anti-α-tubulin mAb DMI A were purchased from Sigma. Anti-p150,glued mAb (the largest subunit of dynactin) was purchased from BD Transduction.

**Plasmid Construction and Fusion Protein Production**—cDNAs were generated by restriction digestion of 4.1R cDNAs (34) or by PCR using an amplification kit (Promega Corp.) and a thermocycler (PCR System 480, PerkinElmer Life Sciences). The 135-kDa GST fusion construct was generated by cloning a human 4.1R cDNA containing all exons (35) from Sigma. Anti-p150 glued mAb (the largest subunit of dynactin) was purchased from BD Transduction.

**Recombinant GST-4.1R was produced according to the manufactur- ing and purification of 4.1R were accomplished by isolation of 4.1Rs from recombinant 4.1R protein was added to an equilibrium dialysis containing cytochalasin B and protease inhibitors. Cells were Dounce- homogenized, and the crude extract was subjected to ultracentrifuga- tion at 100,000 × g for 15 min at 4 °C. The supernatant was concentrated, and laurulin B (5 μM/ml) was added to reduce actin polymerization and the contamination of microtubule pellets with actin and actin-associated proteins. The supernatant was then supplemented with 10 μM taxol and 2.5 μM ATP, and microtubule asters were assembled by incubation at 30 °C for 60 min. After incubation, samples were pro- cessed as described previously (36). The remaining samples were centrifuged through 50% sucrose cushions prepared in KMH at 100,000 × g for 2 h at 4 °C to collect mitotic microtubule-associated proteins in the pellet. Both supernatant and insoluble pellet were collected directly in Laemmli sample buffer for Western blot analysis.

**Immunodepletion and Reconstitution Assay**—Irradiated B-treated HeLa mitotic extracts described above were subjected to immunodepletion. Thirty micrograms of pre-immune rabbit IgG or 4.1R affinity-purified anti-HP antibodies were coupled to protein A beads and incubated with mitotic extracts for 20 min at 4 °C. The beads were then washed, and the supernatant was added to Western blot analysis.

**Comunoprecipitation and Immunoblotting**—Comunoprecipitation of 4.1R, tubulin, and NuMA was performed using HeLa mitotic extracts as described previously (20). In brief, mitotic HeLa cells were homogenized in communoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 5 mM KCl, 1 mg/ml bovine serum albumin, 0.5 mM benzamidine, 1 mM iodoacetamide, 2% CHAPS, protease inhibitors, phosphatase inhibitors), and given 20 strokes in a tight-fitting glass homogenizer. The homogenate was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was pre-cleared with pre-immune rabbit IgG. Subse-
Results

Protein 4.1R in Microtubule Aster Assembly—The co-localization of 4.1R and tubulin at the spindle and spindle poles of mitotic cells prompted us to investigate whether mitotic 4.1R isoforms interact with microtubules. Our analysis of 4.1R mRNA expression in HeLa cells indicates that a 135-kDa isoform (4.1R135), containing all of the alternatively spliced exons except exons 3, 14, 15, 17a, and 17b, is a predominant isoform that is expressed in mitotic cells. To determine whether 4.1R135 interacts directly with microtubules, we used purified recombinant 4.1R135 protein and pure microtubules in microtubule sedimentation assays to analyze the interaction between 4.1R135 and tubulin. An 80-kDa 4.1R isoform (4.1R80) that has been shown to interact with microtubules (38) served as a positive control. Taxol-polymerized microtubules were incubated with recombinant 4.1R135 or 4.1R80 and sedimented through a 50% glycerol cushion. Although 4.1R135 and 4.1R80 alone did not sediment to the pellet (data not shown), both forms were readily detected in the pellet fraction with polymerized microtubules (Fig. 2B, pellet) but not in the supernatant fraction (Fig. 2B, sup), suggesting their interaction with microtubules in vitro. This interaction was specific: a well characterized MAP2 was bound to microtubules, whereas bovine serum albumin did not bind (data not shown).

To confirm the specificity of the interaction between 4.1R and native tubulin in mitotic cells and to identify the domains of 4.1R responsible for the interaction, we performed a GST pull-down assay using homogenates of mitotic HeLa cells. We expressed either the full-length (135 and 80 kDa) of 4.1R or its individual domain (HP, MBD, 16 kDa, SAB, and CTD) (Fig. 2A) as GST fusion proteins (Fig. 2C). GST-HP migrated with an apparent molecular mass of ~80 kDa instead of the calculated ~55 kDa, most likely because of post-translational modification. Equivalent amounts of the GST fusions and control GST were bound to glutathione matrices and incubated with homogenates of HeLa mitotic lysates. After extensive washing, we used Western blotting to examine the matrix-bound GST fusion proteins for their ability to absorb native tubulin. Consistent with the in vitro sedimentation analysis, this blotting revealed that tubulin was specifically associated with the GST-4.1R135 and GST-4.1R80 (Fig. 2D). When individual domains were tested, only GST-MBD and GST-CTD specifically retained native tubulin (Fig. 2D), suggesting that the MBD and CTD of 4.1R are responsible for its binding to tubulin in mitotic HeLa cells. The binding between the MBD or CTD with native mitotic tubulin was specific in that GST alone did not interact with tubulin. At this time, we do not know what type of post-translational modification is involved in GST-HP. However, it appears that the modification at the HP region does not contribute to the tubulin binding ability of 4.1R, because both the 80-kDa isoform, without the HP, and the 135-kDa isoform, with the addition of the HP, bind to tubulin efficiently.
Protein 4.1R Is a Component of Mitotic Asters Assembled in a Cell-free Mitotic Extract—To determine whether 4.1R plays an active role in organizing the mitotic spindle assembly, we first asked whether 4.1R is a component of mitotic asters assembled in a well established in vitro mitotic aster assembly system (25, 30, 32). In addition to the spectrin-actin-4.1R ternary complex interaction (39, 40), 4.1R exhibits a binary interaction with spectrin (39, 41) or actin (42). We reduced actin polymerization and eliminated the possible interaction between actin and 4.1R by the addition of latrunculin B to mitotic extracts prior to the assembly process. Microtubules were induced to polymerize with 10 µM taxol in the presence of 2.5 mM ATP in the HeLa mitotic extract. Under incubation at 30 °C for 60 min, mitotic asters organized into aster-like arrays in a centrosome-independent process that is driven by microtubule motors and structural proteins. Mitotic asters assembled in this extract are composed of microtubules arranged in a radial array that contain NuMA at the central core (Fig. 3A, +ATP) (25). Consistent with the report of Gaglio and coworkers (33), the microtubules were arranged in poorly organized aggregates rather than aster arrays in the absence of ATP (Fig. 3A, −ATP). NuMA did not concentrate at the central core in these aggregates.

We separated the asters from the soluble components of the extract by centrifugation through a 50% sucrose cushion, which allows for the sedimentation of microtubule asters with sufficient mass (32). Under this condition, a portion of 4.1R, but not actin, was co-sedimented with NuMA, dynactin, and tubulin (Fig. 3B, lane P); the latter three proteins are known to associate with microtubules in the microtubule pellet. The inclusion of latrunculin B and the use of 50% sucrose cushions eliminated the contamination of actin in the insoluble pellet (Fig. 3B, actin) and ruled out the possibility of 4.1R interacting with actin. These results demonstrate that 4.1R is a mitotic microtubule-associated protein.

The efficiency of the aster formation is calculated as the conversion of both NuMA and tubulin from a soluble form to an insoluble form (33) as judged by the amount of NuMA and tubulin sedimented into the pellet of the 50% sucrose cushion. It is worth noting that there is a high correlation between the morphology of the aster and the efficiency of aster formation. We standardized our assay conditions as described in the “Experimental Procedures” section and analyzed the asters formed from extracts that exhibit assembly efficiencies ranging between 70 to 85%. With such efficiency, we usually observed ~20 clear asters per field under a ×100 oil objective. In each separate experiment, we viewed ~20 fields. Thus, a total of ~400 asters examined display approximately the same size and morphology as shown in Fig. 3A.

Protein 4.1R Is Required for Mitotic Aster Assembly in Vitro—To determine whether the formation of mitotic microtubule asters in synchronized mitotic HeLa extracts requires 4.1R, we examined whether the assembly of mitotic asters was perturbed by the depletion of 4.1R from the extract. In three successive depletions prior to the assembly reaction, the extracts were immunodepleted with 30 µg of affinity-purified anti-HA antibodies or purified pre-immune rabbit IgG. We used Western blotting to examine the degree to which 4.1R, as well as its associated proteins NuMA and tubulin, were depleted from the extracts. Pre-immune IgG did not immunodeplete any 4.1R, NuMA, or tubulin from the cell lysate, because...
Fig. 4. Depletion of 4.1R from mitotic extract inhibited mitotic aster assembly in vitro. A, Western blot analysis of immunodepleted mitotic extracts. Extracts were successively depleted three times with 30 μg of pre-immune IgG or anti-HP antibodies. The degree of 4.1R depletion and co-depletion of NuMA and tubulin was determined by the presence of its respective protein in protein A-Sepharose-bound fractions as indicated (protein A-Sepharose-bound fractions are indicated by P-1, P-2, and P-3; unbound fractions by Sup-1, Sup-2, and Sup-3). For control, 25 μg of mitotic cell lysates was loaded. B, efficiency of aster assembly in mitotic extracts immunodepleted with rabbit pre-immune IgG (Pre-immune) and anti-HP IgG (4.1R-depleted). The immunodepleted extracts were used to assemble asters in vitro. The same volumes of reaction mixtures were placed on coverslips and processed for indirect immunofluorescence using anti-α-tubulin (green) and anti-NuMA (red) antibodies. The merged yellow color indicates the superimposition of tubulin and NuMA. Bar, 5 μm.

Each successive depletion showed that these proteins were absent in protein A-Sepharose-bound fractions (Fig. 4A, Pre-immune depleted, lanes P-1, P-2, and P-3). Analysis of the supernatant fractions confirmed that 4.1R, NuMA, and tubulin were present in the supernatant in all three pre-immune IgG depletions (Fig. 4A, Pre-immune depleted, lanes Sup-1, Sup-2, and Sup-3). Anti-HP antibodies efficiently depleted 4.1R and ~5% each of NuMA and tubulin in the first depletion (Fig. 4B, 4.1R-depleted, lane P-1). The second depletion removed the remaining ~5% of 4.1R and a small fraction of NuMA as well as tubulin (Fig. 4A, 4.1R-depleted, lane P-2). The third depletion did not remove any 4.1R, NuMA, or tubulin (Fig. 4A, 4.1R-depleted, lane P-3). 4.1R was completely absent from the final supernatant, whereas ~95% of NuMA and tubulin were still present in the final supernatant (Fig. 4A, 4.1R-depleted, lane Sup-3). The in vitro aster assembly was carried out using the supernatant from the third immunoprecipitation.

We spotted equal amounts of assembly reactions from pre-immune-depleted and 4.1R-depleted extracts on coverslips and co-stained them for α-tubulin and NuMA to examine the efficiency of aster assembly. Three separate aster assemblies were performed. Treatment of the extracts with pre-immune rabbit IgG did not alter either the morphology of the mitotic asters or the distribution of the NuMA protein at the core of these structures (Fig. 4B, Pre-immune). The asters assembled from the pre-immune depleted lysates have similar size and morphology to the untreated control lysates. The assembly efficiency is also comparable to that of the control, in which ~20 asters were observed per field. We routinely examined ~400 asters per experiment.

However, depletion of 4.1R from the extract with the anti-HP antibody inhibited the assembly of the mitotic asters and yielded randomly arranged microtubules (Fig. 4B, 4.1R-depleted). There were no visible organized asters in all three experiments performed. The failure of aster assembly in the 4.1R-depleted extracts was apparently not due to indirect depletion of NuMA or α-tubulin, because Western blotting showed that the amounts of NuMA and α-tubulin were comparable in the control (Fig. 4A, Pre-immune depleted, lane Sup-3) and experimental (Fig. 4A, 4.1R-depleted, lane Sup-3) extracts. These results demonstrated that organization of microtubules into asters required 4.1R or a 4.1R-containing complex in a cell-free system.

To determine whether 4.1R is the functional component depleted from the extract using the anti-HP antibody, we tested whether the purified recombinant 4.1R protein would reconstitute mitotic aster formation in the 4.1R-depleted extracts. We first verified the amount of 4.1R to be added into the reconstruction assay by examining the number of copies of 4.1R135 in a single mitotic cell. For this reason, we immunoblotted known amounts of recombinant 4.1R-HP and serial dilutions of mitotic HeLa extract with anti-HP antibody (Fig. 5A). We used Photoshop to scan the anti-HP chemiluminesograms and quantified the protein bands with the NIH Image software for the Macintosh computer. We estimate that each mitotic HeLa cell has ~5 X 105 copies of 4.1R135 (Fig. 5A). The amount of recombinant 4.1R135 at a concentration of 0.35 μg is equal to that of endogenous 4.1R135 from 3 X 105 mitotic HeLa cells.

The reconstitution assays were performed using extracts isolated from 3 X 107 mitotic HeLa cells that have been subjected to immunodepletion with anti-HP antibodies. Supplementation of the 4.1R-depleted extract with purified recombinant 4.1R135 at concentrations equivalent to 1- to 10-fold of endogenous 4.1R135 restored mitotic aster formation (Fig. 5B, 1X). The reconstituted assembly reactions exhibited ~75% assembly efficiency and resulted in ~20 asters per field. The addition of recombinant 4.1R135 to the 4.1R-depleted extract at quantities exceeding normal endogenous levels (5 times and 10 times molar excess) did not appear to increase the number of aster formations or alter the morphology of the asters (Fig. 5B, 5X and 10X). When centrifuged through a 50% sucrose cushion, recombinant 4.1R135 was recovered along with NuMA and tubulin from the mitotic microtubule pellets (Fig. 5C). These results suggest that a 135-kDa 4.1R isoform is required for mitotic aster formation in a cell-free system.
IgG did not precipitate any immunoreactive band (Fig. 6A) on a Western blot using anti-or anti-NuMA antibodies. Analysis of a duplicated co-precipitation assay into soluble (S) and insoluble (P) fractions and subjected to immunoblotting using anti-4.1R antibodies, whereas anti-HP antibodies showed a strong 135-kDa immunoreactive band from anti-tubulin immunoprecipitates (Fig. 6, upper panel). Consistent with our previous report (20) that a 135-kDa 4.1R isoform associates with NuMA in a mitotic complex, anti-exon 13 detected a 135-kDa 4.1R in NuMA immunoprecipitates (Fig. 6D, α-exon 13, lane α-NuMA, upper panel). Analysis of anti-tubulin immunoprecipitates with the anti-exon 13 Ab (Fig. 6D, α-exon 13, lane α-tubulin, upper panel) revealed an immunoreactive band of 135 kDa. These data further suggest that a 135-kDa isoform of 4.1R interacts with tubulin and NuMA in mitotic HeLa cells.

The results obtained so far suggest that a small fraction of a 135-kDa 4.1R isoform occurs in vivo in a complex with a small fraction of the mitotic apparatus proteins tubulin and NuMA. To estimate what fractions of 4.1R and tubulin associate together, we determined the efficiencies of immunoprecipitation and co-precipitation by analyzing both the protein A Sepharose-bound (Fig. 6, Bound) and unbound (Fig. 6, Unbound) fractions. We used cell lysates from 10^7 mitotic cells with 10 μg each of anti-HP, anti-a-tubulin, and anti-NuMA IgGs for each immunoprecipitation assay. Quantitation from three separate experiments shows that ~80% of 4.1R was precipitated by anti-HP Ab that co-precipitated ~1% of tubulin and that ~50% of tubulin was precipitated by anti-tubulin mAb, which in turn brought down ~5% of 4.1R. Consistent with our previous report (20), 4.1R and NuMA co-immunoprecipitated with limited efficiency; ~50% of NuMA was precipitated by anti-NuMA mAb that brought down ~5% of 4.1R. The lower efficiency of co-immunoprecipitation compared with immunoprecipitation suggests that only a fraction of these molecules associate together in vivo. This is consistent with our immunofluorescent staining results, suggesting that subpopulations of 4.1R and tubulin partially co-localize together.

**DISCUSSION**

Protein 4.1R has been known to localize to the spindle and spindle poles of mitotic cells (17, 19, 20); however, little is known about the topography and relation of 4.1R to the major constituent of the mitotic apparatus, the microtubules, or about its function in spindle/spindle poles. In this report, we demonstrate that 4.1R co-localizes with microtubules at the mitotic spindle and spindle poles, co-sediments with microtubules in vitro, co-immunoprecipitates with tubulin in vivo, and associates with tubulin in mitotic HeLa cell extracts through its MBD and CTD. Furthermore, we show that 4.1R is a mitotic microtubule-associated protein and is essential to mitotic aster assembly in vitro in a cell-free extract prepared from synchronized HeLa cells. Based on these results, we propose that non-erythroid 4.1R may contribute to the organization of mitotic spindles.

The cell cycle-dependent localization of 4.1R is unique; it localizes to the nucleus and cytoplasm during interphase and redistributes to the spindle poles during mitosis, at which point...
4.1R partially co-localizes with two major constituents of the mitotic apparatus, NuMA, and microtubules. We (20) previously showed that 4.1R directly interacts with NuMA through its C-terminal domain and forms a complex with the spindle pole-organizing proteins NuMA, dynein, and dynactin during cell division. We now show that, in addition to its interaction with NuMA, 4.1R interacts with tubulin in mitotic cells and is a mitotic microtubule-associated protein. It has been previously documented that purified 4.1R from red blood cells interacts specifically with the C-terminal domain of tubulin (38). In human T cells, 4.1R isoforms lacking exon 16 co-localize and interact with interphase microtubules through a 22-amino acid sequence located within exon 10 of the MBD domain (13). Consistent with these reports, our immunofluorescent staining showed a partial co-localization of 4.1R and tubulin in interphase HeLa cells, and in vitro sedimentation experiments confirmed the interaction of an erythrocyte 4.1R isoform with purified microtubules. Moreover, we showed that a major HeLa 4.1R isoform containing all of the alternatively spliced exons except exons 3, 14, 15, 17a, and 17b also sedimented with purified microtubules. Furthermore, we demonstrated that both 4.1R and 4.1R isoforms associate with tubulin in a GST pull-down assay using HeLa mitotic extracts. In contrast to the interaction of 4.1R isoforms with interphase T-cell tubulin (13), an interaction that requires only the MBD, we found that both the MBD and the CTD of 4.1R contribute to its binding to tubulin in HeLa mitotic extracts. Post-translational modifications of tubulin have been shown to regulate its association with microtubule-associated proteins (43). Polyglutamylated tubulin was detected in proliferating cells of different origins (HeLa, KE37, and NIH 3T3), where it associates with the centrosomes, the spindle, and the midbody (44). The localization of 4.1R coincides with that of tubulin during mitosis; thus, it is possible that the post-translational modification of tubulin in mitotic cells might facilitate their selective recruitment of 4.1R isoforms through their MBD as well as their CTD into distinct microtubule populations, hence modulating their functional properties.

Alternatively, a third protein may be involved in the indirect interaction of mitotic tubulin and the CTD of 4.1R. A good candidate protein is NuMA, because NuMA interacts with tubulin through amino acids 1868–1967 (37) and with the CTD of 4.1R through amino acids 1788–1810 (20). This suggests that the same population of NuMA may link 4.1R molecules to tubulin; thus, these proteins can form a three-way complex. Using a complementary assay, co-immunoprecipitation, we also demonstrated that 4.1R appears to interact in vivo with NuMA as well as with tubulin. However, binary interactions between NuMA and tubulin also occur; the depletion of 4.1R from the mitotic HeLa extracts did not dissociate the interaction of NuMA and tubulin, as shown through their association in randomly dispersed microtubules assembled in 4.1R-immune-depleted extracts (see Fig. 4B, 4.1R-depleted). Further experiments are required to determine whether 4.1R associates with NuMA and tubulin simultaneously or individually. Nevertheless, the association of 4.1R, NuMA, and tubulin suggests that 4.1R may play a role in organizing mitotic spindles.

Centrosome-free spindle-pole formation that depends on the action of non-centrosomal structural and microtubule motor proteins was directly demonstrated during spindle assembly in extracts from metaphase-arrested frog eggs (45). Dynein-dependent and centrosome-free spindle-pole formation was mimicked by the induction of microtubule asters in the absence of the drug taxol in both Xenopus (46) and mammmalian systems (25). An in vitro aster assembly assay using HeLa mitotic extracts, an assay that recapitulates many of the structural events in mitosis, has been used widely to characterize the function of a number of proteins localized at the spindle poles (29–33). We used this assay to show that mitotic aster assembly was inhibited in a 4.1R depletion-specific manner and that assembly could be re-initiated with purified recombinant 4.1R isoforms. These results suggest that 4.1R is essential to the assembly of mitotic asters in vitro.

Our immunoprecipitation/co-precipitation assays showed that only a subpopulation of NuMA and α-tubulin interacts with 4.1R. Consistent with these results, the immunodepletion assays further confirmed that 4.1R antibodies failed to co-deplete significant amounts of NuMA and α-tubulin. These results imply that aster assembly is not abolished because of the lack of NuMA or α-tubulin. This idea is further supported by the observation that NuMA still associates with the irregularly shaped microtubules in the 4.1R-depleted assembly reactions. Thus, depletion of 4.1R does not seem to affect the association of NuMA with tubulin. Rather, recruitment of 4.1R to the astral arrays by tubulin and/or NuMA could be functionally necessary for assembly or maintenance of astral arrays.

The biological significance of 4.1R in centrosome-dependent spindle-pole formation in somatic cells is unclear. 4.1R might
stabilize the interaction among NuMA, dynein, dynactin, and microtubules in a manner analogous to its role in the stabilization of the association of spectrin, actin, and integral proteins in red blood cells. Viable homozygous 4.1R knockout mice have been generated (47). The viability of these mice is perplexing, given the wide tissue distribution of 4.1R and its possible involvement in cell division. However, it is not unprecedented that germ line alterations that activate or inactivate genes of interest might have effects other than somatic mutations because of developmental compensation (48, 49). For example, the acute loss of Rb in primary quiescent cells has phenotypic consequences that differ from germ line loss of Rb function (50).

In cell division, the function of 4.1R in 4.1R null mice may be more, small interference RNA-caused knockout of 4.1R in HeLa cells also resulted in poorly focused spindle poles in mitotic cells (51). In this regard, the assumption of 4.1R is necessary to assess the possible functional redundancy of these 4.1 family members. Regardless, the ability of 4.1R to interact with mitotic tubulin and the fact that mitotic aster assembly in vitro requires 4.1R suggest that 4.1R may be pivotal to the structural organization and maintenance of the mitotic apparatus during cell division. This idea is supported by an in vitro aster assembly assay using Xenopus egg extracts (51) that indicates that the SAB and CTD peptides significantly deranged the normal symmetrical microtubule array and dispersed the tight focus of NuMA. Furthermore, small interference RNA-caused knockout of 4.1R in HeLa cells also resulted in poorly focused spindle poles in mitotic cells (51). Additional work will establish the precise role of cytoskeletal 4.1R in the mechanochemistry of the mitotic spindle. This report, however, suggests that 4.1R is crucial and is related to mitotic tubulin binding.

Acknowledgments—We thank Julia Hartenstein and Dr. Siu-Hong Chan for their early studies that contributed to this work. We acknowledge Indira Munagala for her assistance with the analysis of number edge Indira Munagala for her assistance with the analysis of number

REFERENCES

1. Benz, E. J., Jr. (1994) in The Molecular Basis of Blood Diseases (Stamatakopulos, G., Neinhuis, A. W., Majerus, P., Varmus, H., eds) 2nd Ed., pp. 257–292

2. Parra, M. K., Gei, S. L., Koury, M. J., Mohandas, N., and Conboy, J. G. (2003) Blood 101, 4164–4171

3. Conboy, J. G., Chan, J., Mohandas, N., and Kan, Y. W. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9062–9065

4. Tang, T. K., Quin, Z., Marchesi, V. T., and Benz, E. J., Jr. (1990) J. Cell Biol. 110, 617–624

5. Huang, S. C., Iakoubov, F., Tang, T. K., and Benz, E. J., Jr. (1992) Trans. Assoc. Am. Phys. Vol. CV, 165–171

6. Chais, J. A., Coulombel, L., McGee, S., Lee, G., Tchernia, G., Conboy, J. G., and Mohandas, N. (1996) Blood 87, 5324–5331

7. Subrahmanyan, G., Bertics, P. J., and Anderson, R. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5222–5226

8. Walemsky, L., Blackshaw, S., Liao, D., Watkins, C. C., Weier, H. U., Parra, M., Hoghair, R. L., Conboy, J. G., Mohandas, N., and Snyder, S. H. (1999) J. Neurosci. 19, 6457–6467

9. Obara, R., Yakamaw, H., Nakayama, M., Yuasa, S., and Obara, O. (1999) Brain Res. Dev. Brain Res. 117, 127–138

10. Tran, Y. K., Bogler, O., Gorse, K. M., Wieland, I., Green, M. H., Newsham, I. F. (1999) Cancer Res. 59, 35–43

11. Parra, M., Gascard, P., Walemsky, L., D., Gim, J. A., Blackshaw, S., Chan, N., Takakuya, Y., Berger, T., Lee, G., Chais, J. A., Snyder, S. H., Mohandas, N., and Conboy, J. G. (2000) J. Biol. Chem. 275, 3247–3255

12. Ramez, M., Blot-Chabaud, M., Cluzeaud, F., Chanan, S., Patterson, M., Walemsky, L., D., Marfatia, S., Baines, A. J., Chais, J. A., Conboy, J. G., and Mohandas, N., and Gascard, P. (2003) Kidney Int. 63, 1221–1337

13. Fincke, M., Loque, C. M., and Conboy, J. G., and Conboy, J. G. (2001) J. Biol. Chem. 276, 44785–44791

14. Kontrogianni-Kostantopoulos, A., Huang, S. C., and Benz, E. J., Jr. (2000) Mol. Cell Biol. 21, 3865–3871

15. Mattagajasingh, S. N., Huang, S. C., Hartenstein, J. S., and Benz, E. J., Jr. (2000) J. Biol. Chem. 275, 30573–30585

16. De Carcer, G., Lallena, M. J., and Conboy, J. G. (1998) Biochem. J. 322, 871–877

17. Hara, N. J., and Merdes, A. (2002) J. Cell Biol. 158, 137, 74 Springer-Verlag, New York
Protein 4.1R, a Microtubule-associated Protein Involved in Microtubule Aster Assembly in Mammalian Mitotic Extract
Shu-Ching Huang, Ramasamy Jagadeeswaran, Eva S. Liu and Edward J. Benz, Jr.

J. Biol. Chem. 2004, 279:34595-34602.
doi: 10.1074/jbc.M404051200 originally published online June 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404051200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 33 of which can be accessed free at http://www.jbc.org/content/279/33/34595.full.html#ref-list-1