Mitotic Regulation of Protein 4.1R Involves Phosphorylation by cdc2 Kinase

Shu-Ching Huang,^{*†‡} Eva S. Liu,^{*} Siu-Hong Chan,[§] Indira D. Munagala,^{*} Heidi T. Cho,^{*} Ramasamy Jagadeeswaran,^{*} and Edward J. Benz Jr.^{*†||¶#}

^{*}Department of Medical Oncology, Dana Farber Cancer Institute, Boston, MA 02115; Departments of [†]Medicine and [‡]Pathology, Harvard Medical School, Boston, MA 02115; [§]Department of Medicine, Johns Hopkins University, Baltimore, MD 21205; ^{||}Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115; and [¶]Department of Pediatrics, Children's Hospital of Boston, Boston, MA 02115

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The nonerythrocyte isoform of the cytoskeletal protein 4.1R (4.1R) is associated with morphologically dynamic structures during cell division and has been implicated in mitotic spindle function. In this study, we define important 4.1R isoforms expressed in interphase and mitotic cells by RT-PCR and mini-cDNA library construction. Moreover, we show that 4.1R is phosphorylated by p34^{cdc2} kinase on residues Thr60 and Ser679 in a mitosis-specific manner. Phosphorylated 4.1R¹³⁵ isoform(s) associate with tubulin and Nuclear Mitotic Apparatus protein (NuMA) in intact HeLa cells in vivo as well as with the microtubule-associated proteins in mitotic asters assembled in vitro. Recombinant 4.1R¹³⁵ is readily phosphorylated in mitotic extracts and reconstitutes mitotic aster assemblies in 4.1R-immunodepleted extracts in vitro. Furthermore, phosphorylation of these residues appears to be essential for the targeting of 4.1R to the spindle poles and for mitotic microtubule aster assembly in vitro. Phosphorylation of 4.1R also enhances its association with NuMA and tubulin. Finally, we used siRNA inhibition to deplete 4.1R from HeLa cells and provide the first direct genetic evidence that 4.1R is required to efficiently focus mitotic spindle poles. Thus, we suggest that 4.1R is a member of the suite of direct cdc2 substrates that are required for the establishment of a bipolar spindle.

INTRODUCTION

Erythrocyte protein 4.1 (4.1R) is an 80-kDa cytoskeletal phosphoprotein critical to the dynamic organization of the spectrin/actin cytoskeleton and to the attachment of the cytoskeleton to the cell membrane (reviewed by Benz, 1994). The 80-kDa 4.1R of mature red blood cells is only one member of a large 4.1R family that arises from a single gene through the utilization of alternative promoters (Parra *et al.*, 2003), alternative pre-mRNA splicing (Conboy *et al.*, 1988; Tang *et al.*, 1990), and posttranslational modifications (Subrahmanyam *et al.*, 1991). Three 4.1R homologues, named 4.1G, 4.1N, and 4.1B, have been identified (Parra *et al.*, 1998; Walensky *et al.*, 1999; Yamakawa *et al.*, 1999; Ye *et al.*, 1999; Parra *et al.*, 2000). They share strong sequence homology with the 30-kDa membrane-binding domain (MBD), the 10-kDa spectrin/actin-binding (SAB) domain, and the 22–24-kDa C-terminal domain (CTD) of 4.1R. The NH₂ terminus (head-piece or HP) and intervening sequences appear to be highly specific for each individual 4.1 gene. Several research studies are investigating the functional specificity and redundancy of these proteins.

Isoforms of 4.1R are widely expressed in many tissues and are phylogenetically conserved (Cohen *et al.*, 1982; Granger and Lazarides, 1984), which imply their importance in cell

function. Studies of 4.1R have revealed unexpected new roles for 4.1R proteins in various supramolecular structures in nucleated cells. In contrast to 4.1R's strict peripheral localization in mature red blood cells, 4.1R isoforms have been shown to associate with interphase microtubules in human T-cells (Perez-Ferreiro *et al.*, 2001), contractile apparatus in skeletal muscles (Kontogianni-Konstantopoulou *et al.*, 2000), and tight junctions in epithelial cells (Mattagajasingh *et al.*, 2000). Isoforms of 4.1R in the nucleus have also been shown to contribute to nuclear architecture (DeCarcner *et al.*, 1995; Krauss *et al.*, 1997a, 2002, 2003; Mattagajasingh *et al.*, 1999) and splicing processes (Lallena and Correia, 1997).

A unique feature of 4.1R is that it exhibits dynamic reorganization during the cell cycle (Mattagajasingh *et al.*, 1999; Krauss *et al.*, 1997a). 4.1R localizes in the centrioles, nucleus, and cytoplasm of interphase cells, and in the spindle and spindle poles of mitotic cells. Previously, we have shown that a 135-kDa 4.1R isoform associates in a complex containing NuMA, cytoplasmic dynein, and dynactin (Mattagajasingh *et al.*, 1999), which is involved in the tethering of microtubules at the spindle poles and is essential for mitotic spindle-pole stabilization (Merdes *et al.*, 1996). 4.1R has also been shown to be crucial for in vitro mitotic aster assemblies in both *Xenopus* egg extracts (Krauss *et al.*, 2004) and mammalian mitotic extracts (Huang *et al.*, 2004). The cell cycle-dependent localization of 4.1R and its association with microtubule-associated proteins strongly imply a critical role for 4.1R in the organization of the mitotic apparatus during cell division. However, little is known about the expression of 4.1R isoforms and the mechanisms by which it reorganizes during the cell cycle.

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† Corresponding author. E-mail address: shu-ching_huang@dfci.harvard.edu.

The relocalization pattern of 4.1R during the cell cycle is similar to that of several proteins essential for mitotic spindle assembly, including NuMA (Price and Pettijohn, 1986), dynein (Steuer *et al.*, 1990), HSET (Kuriyama *et al.*, 1995), and Eg5 (Houlston *et al.*, 1994). Many of the cellular reorganizations occurring at the onset of mitosis are triggered, directly or indirectly, by the activation of p34^{cdc2} kinase (reviewed by Morgan, 1995). These include multiple proteins involved in spindle assembly (NuMA and Eg5; Blangy *et al.*, 1995; Compton and Luo, 1995), chromosome segregation (CENP-E; Liao *et al.*, 1994), and mitotic disassembly of the nuclear lamina (Peter *et al.*, 1990). Three predicted p34^{cdc2} consensus phosphorylation sites in the 4.1R protein are conserved across many species, which suggests that posttranslational modification by p34^{cdc2} may be one mechanism for the spatial and temporal regulation of 4.1R's activity during the cell cycle. In this study, we identified the predominant 4.1R isoforms expressed and demonstrated a p34^{cdc2} kinase phosphorylation-based regulation of 4.1R during cell division. We further showed that depletion of 4.1R by siRNA resulted in defective metaphase and multinuclei. Our results suggest that the phosphorylation of 4.1R may establish its interactions with NuMA and microtubules and play an important role in mitotic spindle pole organization.

MATERIALS AND METHODS

Cell Culture and Cell Synchronization

HeLa cells were maintained in DMEM plus 10% fetal bovine serum in a 5% CO₂ incubator at 37°C. The cells were synchronized at the G1/S phase boundary using the thymidine double-block method (O'Connor and Jackman, 1995). After the release from the second thymidine block, the cells were allowed to grow for 4 h. Nocodazole was then added to a final concentration of 60 ng/ml. The mitotic cells accumulated over the next 6 h were collected by mitotic shake off. Other stages of the cell cycle were also collected: G0 cells were collected after 24 h of serum starvation, S phase cells were collected 2 h after being released from the thymidine block, and G2 phase cells were collected by treating cells with nitrogen mustard as described (Maity *et al.*, 1996). Roscovitine (100 μ M; Sigma, St. Louis, MO), used to inhibit cyclin-dependent kinase activity, was added to mitotic cells released from the G1/S phase.

HeLa Tet-Off cells were purchased from Clontech Laboratories (Palo Alto, CA). 4.1R¹³⁵/GFP/pTRE and 4.1R^{135(T60A,S679A)}/GFP/pTRE were stably co-transfected with pTK-Hyg (Clontech) into HeLa Tet-Off cells using Lipofectamine (Invitrogen, Carlsbad, CA) transfection reagent. Doxycycline at a concentration of 100 ng/ml, which induces levels of exogenous 4.1R expression similar to that of endogenous 4.1R, was used for 4.1R expression. siRNA duplexes of 4.1R and the control GL2 luciferase (Dharmacon Research, Boulder, CO) were transfected into HeLa cells using Oligofectamine (Invitrogen).

Preparation of Plasmids and Fusion Proteins

Complementary DNAs were generated using a full-length 4.1R cDNA (Tang *et al.*, 1988) as the template by the PCR. Constructs used for expression of 4.1R-glutathione S-transferase (GST) fusion proteins (HP/GST, 16 kDa/GST) have been described (Mattagajasingh *et al.*, 1999). Construct SAB+CTD/GST was amplified with primer set 5'-GAAAAAAGAGAGAAAGA3' and 5'-TCACTCATCAGCAATCTC3'. The mutation constructs, HP^{T60A}/GST, 16 kDa^{S555A}/GST, and SAB+CTD^{S679A}/GST, were made by two-step PCR as described (Mattagajasingh *et al.*, 1999). Primer sets (with mutated nucleotides underlined) (5'-ATGACAACAGAGAAGAGTTAGTGACT3', 5'-TCATGTGTAGGAGCGTCTCCATT3') and (5'-AATGGAGACGCTCTACACATGA3', 5'-GTTCTGTGTTTCTGATTGGT3') were utilized for the incorporation of Thr60Ala mutation in HP; primer sets (5'-CCGATACAGTGGCCGGACTCAA3', 5'-AGGAGCTCGGTCTGCCAATCGACAGCT3'), 5'-AGACCGAGCTCTCGGCCACTTCTGCACT3', 5'-CCATGCTTCTGTGGGCTCTGG3') were used for the incorporation of Ser555Ala in 16-kDa; primer sets (5'-AAGAAAAAGAGAGAAAGACTA3', 5'-TCGGAAGGGTGGTGAAGTG-GATAAGCGT3') and (5'-TCCACTCACGCACCCCTCCGAA3', 5'-CTCATCAGCAATCTCGGT3') were used for the incorporation of Ser679Ala mutation in SAB+CTD. The resultant second step PCR products were subcloned into pGEX-6P1 (Amersham Pharmacia Biotech, Piscataway, NJ).

The 4.1R¹³⁵/GFP/pTRE and 4.1R^{135(T60A,S679A)}/GFP/pTRE constructs were made by subcloning the sequences 4.1R¹³⁵/GFP and 4.1R^{135(T60A,S679A)}/GFP into pTRE vector (Clontech). The 4.1R¹³⁵/pET31b(+) construct was generated by cloning a human 4.1R cDNA containing all exons (Baklouti *et al.*, 1997) except exons 3, 14, 15, 17a/a' and 17b into pET31b(+) (Novagen, Madison,

WI). The 4.1R^{135(T60A,S679A)}/pET31b(+) has the same exon composition as that of 4.1R¹³⁵/pET31b(+), with the exception of the mutation of both Thr60 and Ser679 to alanine. Accuracy of the reading frame and authenticity of all mutations were confirmed by sequencing.

Recombinant GST-4.1R was produced according to the manufacturer's protocol (Amersham Pharmacia Biotech). When vector pET-31b(+) was used, the cDNA was transformed into *Escherichia coli* BL21 for protein production. The expression and purification of recombinant proteins was as described (Huang *et al.*, 2004). Purified recombinant 4.1R was concentrated using Centricon Plus-20 (Millipore, Bedford, MA). The protein contents were determined using a standard BCA assay determination kit (Pierce Chemical Co., Rockford, IL).

Antibodies

Antibodies to the erythroid 80-kDa 4.1R protein or the recombinant HP of 4.1R (anti-HP) were described (Mattagajasingh *et al.*, 1999). Polyclonal anti-phospho-Thr⁶⁰ specific antibody was generated by immunizing rabbits with the synthetic peptide KASNGDTPPTHTEDLTKN that corresponds to the amino acids 54–69 of human 4.1R sequences with a phosphothreonine at position 60 (TP). The antibody was purified from serum by two rounds of affinity chromatography on a phospho-Thr⁶⁰ peptide column followed by a nonphosphopeptide column. Anti- α -tubulin monoclonal antibody (mAb) and antiactin mAb were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-NuMA mAb was purchased from Oncogene Research Products (Cambridge, MA).

Gene-specific Mini-cDNA Library

Five micrograms of RNA from either interphase or mitotic HeLa cells were subjected to RT using a primer located at the 3' untranslated region (TCTA-GAAGAATCTCTTGGTATAAACTCCCA) according to standard experimental protocols. The primers used in the PCR amplification were chosen to anneal to sequences present in exons 1B (5'-GCTGTGCTGTGTCTCACT-GCTGTCA3') or 1C (AGCCGACAGAGGCCCGAGCCTCGGAC; Parra *et al.*, 2003) and exon 21 (TCACTCATCAGCAATCTCGGTCTCTCTG; Baklouti *et al.*, 1997). The PCR products were subcloned into TOPO TA-vector (Invitrogen) and analyzed in a DNA dot blot assay using 4.1R exon-specific digoxigenin-labeled oligonucleotide probes as described (Kontogianni-Konstantopoulou *et al.*, 2000).

In vivo ³²P-labeling, Kinase, and Dephosphorylation Assays

In vivo ³²P-labeling was performed as described (Compton and Luo, 1995). For immunoprecipitation of 4.1R from ³²P-labeled cells, mitotic and interphase cells were resuspended in immunoprecipitation (IP) buffer (20 mM Tris-HCl, 150 mM NaCl, 2% CHAPS, 0.1% SDS, 1 mg/ml bovine serum albumin, 0.2 mM EDTA, 5 mM iodoacetamide, pH 7.5) containing protease inhibitors and phosphatase inhibitors (5 mM β -glycerophosphate, 10 mM NaF, 10 mM Na₄P₂O₇), and followed the immunoprecipitation procedure described (Mattagajasingh *et al.*, 1999).

In vitro phosphorylation experiments were carried out with purified 4.1R/GST fusion proteins using cdc2 protein kinase (New England BioLabs, Beverly, MA) according to the manufacturer's protocol. Phosphorylated samples were fractionated on SDS-polyacrylamide gels, fixed, dried, and exposed to x-ray film. For dephosphorylation analysis, λ protein phosphatase (New England BioLabs) was added to the anti-HP immunoprecipitates and incubated for 30 min at 30°C. Transfer of proteins to nitrocellulose or PVDF membranes, and detection of 4.1R were carried out by immunoblotting using an ECL detection kit (Amersham Pharmacia Biotech). Some chemiluminograms were scanned using the Adobe Photoshop software (Adobe Systems, San Jose, CA) and the protein bands of interest were quantitated by using the NIH Image software for the Apple Macintosh computer.

In Vitro Mitotic Aster Assembly, Immunodepletion, and Reconstitution Assay

Mitotic extracts were prepared as described (Gaglio *et al.*, 1997; Dionne *et al.*, 2000). In brief, highly enriched mitotic cells collected by mitotic shake off were incubated for 30 min at 37°C with 20 μ g/ml cytochalasin B. The cells were resuspended in KHM buffer (78 mM KCl, 50 mM HEPES, pH 7.0, 4 mM MgCl₂, 2 mM EGTA, 1 mM dithiothreitol) containing 20 μ g/ml cytochalasin B and protease inhibitors. Dounce homogenized, and subjected to ultracentrifugation at 100,000 $\times$ g for 15 min at 4°C. Latrunculin B (5 μ g/ml) was added to the resulting supernatant to reduce actin polymerization and the contamination of microtubule pellets with actin and actin-associated proteins. The final extract was supplemented with 10 μ M taxol and 2.5 mM ATP. Microtubule asters were assembled by incubation at 30°C for 60 min and processed for indirect immunofluorescence microscopy. The remaining extracts containing microtubule asters were layered onto a 50% (wt/vol) sucrose cushion in KHM buffer, and microtubules were collected by sedimentation at 100,000 $\times$ g for 2 h at 4°C. The insoluble pellet was collected directly in SDS/PAGE sample buffer.

Figure 1. 4.1R isoforms expression in interphase and mitotic cells. (A) Schematic representation of the structural domains of 4.1R. These studies used two antibodies, an anti-80-kDa antibody raised against the erythrocyte 4.1R and an anti-HP antibody specific to the region between the AUG-1 and AUG-2 translation initiation sites. (B) Subcellular localization of 4.1R. HeLa cells were fractionated, and 20 μ g of proteins from the cytoplasmic (Cyto.), nuclear (Ncl.), and nuclear matrix (Ncl. Mat.) fractions were analyzed with the anti-HP antibody. (C) The high-molecular-weight 4.1R isoforms exhibit a cell cycle-dependent mobility shift. Lysates of HeLa cells synchronized at the G1/S phase (G1/S) and mitosis phase (M) were separated on SDS-polyacrylamide gels and subsequently probed with anti-80-kDa (left panel) or anti-HP (right panel) antibodies.

Latrunculin B-treated HeLa mitotic extracts described above were subjected to three successive immunodepletions. Thirty micrograms of preimmune rabbit IgG or 4.1R anti-HP antibodies were coupled to protein A beads and incubated with mitotic extracts for 20 min at 4°C. The beads were spun down, the supernatants were collected, and the depletion process was repeated twice. Both beads and supernatants from each depletion were subjected to Western blotting to verify the effectiveness of the depletion of 4.1R. The final supernatants were subjected to the *in vitro* aster assembly. For reconstitution assays, recombinant 4.1R¹³⁵ or 4.1R^{135(T60A, S679A)} at a concentration equal to that of endogenous 4.1R was added to 4.1R-immunodepleted mitotic extracts and subjected to *in vitro* aster assembly.

Indirect Immunofluorescence and Imaging

HeLa cells were grown on poly-D-lysine-coated coverslips and subjected to immunofluorescence staining as described (Mattagajasingh *et al.*, 1999). Mitotic asters assembled *in vitro* were spotted on poly-D-lysine-coated coverslips, fixed in methanol, and subjected to immunofluorescent staining as described (Gaglio *et al.*, 1997). The samples were viewed with a Zeiss Axiovert 200M inverted microscope (Zeiss, Thornwood, NY). The mitotic asters were viewed under an 100 $\times$ oil objective. The images were collected using Slide-Book4 software and processed using Photoshop software (Adobe Systems).

RESULTS

Expression of 4.1R Isoforms during the Cell Cycle

The dynamic localization of 4.1R during the cell cycle has been well documented (Krauss *et al.*, 1997a; Mattagajasingh *et al.*, 1999). 4.1R localizes in the nucleus and in the cytoplasm of interphase cells and distributes to the mitotic spindle and spindle poles during mitosis. However, 4.1R isoforms expression during the cell cycle has not been characterized. We used two 4.1R antibodies (Figure 1A) to characterize 4.1R isoforms expression during the interphase and mitotic stages of HeLa cells: an anti-80-kDa antibody raised against the erythrocyte 4.1R and an anti-HP antibody that is unique to 4.1R. The anti-80-kDa antibody detected both the high-molecular-weight 4.1R isoforms (~135 kDa) translated from the initiator codon AUG-1 located in exon 2' and the low-molecular-weight isoforms (~80 kDa) derived from the initiator codon AUG-2 located in exon 4. The anti-HP antibody detects only 4.1R isoforms derived from the AUG-1 translation site.

We used the anti-HP antibody to examine nuclear, nuclear matrix, and cytoplasmic fractions of interphase HeLa. Anti-HP antibody recognized five immunoreactive bands (~40, 65, 72, 76, and 135 kDa) in the cytoplasmic fraction (Figure 1B, lane Cyto.), two bands (~40 and 135 kDa) in the nuclear fraction (Figure 1B, lane Ncl.), and a 135-kDa band in the nuclear matrix fraction (Figure 1B, lane Ncl. Mat.). The 135-kDa 4.1R isoform is the predominant form in all fractions examined, which suggests that it is the major constituent of 4.1R in the interphase cells.

We then examined whether 4.1R protein expression changes during the cell cycle. Equal amounts of cell lysates from G1/S or mitotic cells were blotted with anti-80-kDa or anti-HP antibody. Anti-80-kDa antibody detected four bands (~78, 80, 135, and 175 kDa) in the G1/S lysate (Figure 1C, anti-80-kDa, lane G1/S) and four bands of similar molecular weight in mitotic lysates (Figure 1C, anti-80-kDa, lane M). The high-molecular-weight 4.1R isoforms (4.1R¹³⁵ and 4.1R¹⁷⁵) from mitotic lysate appeared larger than those from G1/S lysate. However, the 78- and 80-kDa isoforms (4.1R⁷⁸ and 4.1R⁸⁰) retained the same mobility in both interphase and mitotic cells. The anti-HP antibody specifically recognized a 135- and a 175-kDa isoform from G1/S lysate and corresponding retarded forms from mitotic lysate (Figure 1C, anti-HP). The low-molecular-weight forms of 4.1R (4.1R⁷⁸ and 4.1R⁸⁰) could not be detected with anti-HP antibody, which suggests that low-molecular-weight isoforms are derived from the AUG-2. These results further suggest that both 4.1R¹³⁵ and 4.1R¹⁷⁵ isoforms are translated from AUG-1, and the mitotic-specific mobility shift of these isoforms could result from either posttranslational modification or from expression of different isoforms.

The mobility shift of the high-molecular-weight 4.1R isoforms observed in mitotic HeLa lysate prompted us to examine whether the exon composition of 4.1R changes during the cell cycle. 4.1R is comprised of 25 exons within which 10 exons are constitutively spliced and 15 exons are alternatively spliced (Figure 2C). Three exons (exons 1A, 1B, and

Figure 2. Characterization of 4.1R messages in HeLa cells by RT-PCR analysis. (A) Schematic representation of the splicing patterns of exons 1A, 1B, and 1C to exons 2'/2 in 4.1R genes. p3'ss, proximal 3' splice site; d3'ss, distal 3' splice site. (B) Analysis (25 PCR cycles) of 5' exon expression in HeLa cells. Thirty-five amplification cycles were performed for exon 1B. Top: PCR products; bottom: Southern-blot hybridizations using exon 2' as a probe. (C) Diagram and percentile prevalence of the various 4.1R isoforms present in interphase (G1/S) and mitotic (M) cells. The constitutive coding exons are indicated as gray boxes, whereas alternatively spliced exons are depicted as shaded boxes.

1C), located at the 5' untranslated region upstream of exon 2', exhibit differential splicing to exon 2'/2 acceptor sites (Figure 2A; Parra *et al.*, 2003). To characterize 4.1R isoform expressions in HeLa, we first analyzed the 5' untranslated region via RT-PCR. Our analysis of 4.1R in HeLa show that exon 1A splices only to the distal 3' splice site (Figure 2A, d3'ss), excluding exon 2' in mature mRNA, whereas exons 1B and 1C splice to the proximal 3' splice site (Figure 2A, p3'ss), generating a distinctive 5' end containing exon 2'. 4.1R mRNA that includes exon 2' encodes high-molecular-weight isoforms by initiation at an upstream translation site (AUG-1), whereas those excludes exon 2' encodes low-molecular-weight isoforms by initiation at a downstream translation initiation site (AUG-2) that resides in exon 4 (Figure 2C). Although exons 1A and 1C are abundantly expressed in HeLa cells and are readily detected with 25 cycles of PCR amplification, exon 1B was not as abundant and could only be detected when PCR was amplified with 35 cycles (Figure 2B, PCR). Southern blotting of the PCR products with an exon 2' probe confirmed the splicing patterns, in which exon 1A excluded exon 2', whereas exons 1B and 1C included exon 2' (Figure 2B, exon 2').

To examine the exon compositions of the high-molecular-weight 4.1R isoforms, we analyzed the relative abundance of 4.1R splice variants by constructing 4.1R-specific mini-cDNA libraries from both interphase and mitotic HeLa mRNA using a primer set located in exon 1C and exon 21. We identified a repertoire of isoforms in both interphase and mitotic cells (Figure 2C). The most predominant isoform contained all previously identified alternatively spliced exons except exons 3, 14, 15, 17a, and 17b; this isoform accounted for 74% (interphase) and 76% (mitotic) of 4.1R mRNA in HeLa cells (Figure 2C). This isoform encodes for a protein that corresponds to the major 4.1R¹³⁵ identified at the protein level (Figure 1C). 4.1R¹³⁵ is the subject of this study. Additional moderate and minor mRNA species with different exon compositions (Figure 2C) were also detected in HeLa cells. These results suggest that the major interphase 4.1R forms differ from the mitotic forms because of post-translational modification rather than changes in splicing patterns.

4.1R Phosphorylation Is Mitosis specific

To determine whether the mobility shift of 4.1R¹³⁵ and 4.1R¹⁷⁵ isoforms in mitotic phase is regulated by phosphorylation, we synchronized HeLa cells at the G0, G1/S, S, G2, and M phase. We also treated mitotic cells with roscovitine, a selective inhibitor of cyclin-dependent kinase (Schuhmacher *et al.*, 1999). We noticed that anti-HP antibodies detected doublets of ~135- and ~175-kDa forms in some of our immunoblots when we used a different aliquot of HeLa stock. Nonetheless, the doublets also displayed a mobility shift in mitotic cells. The 135-kDa isoform from mitotic cells (Figure 3A, lane M) exhibited an increase in apparent size relative to 4.1R from cells in G0, G1/S, S, and G2 (Figure 3A, lanes G0, G1/S, S, G2). Roscovitine treatment of mitotic cells completely blocked the mitosis-dependent shift in 4.1R mobility (Figure 3A, lane M/Ros), which suggests that 4.1R could not be phosphorylated because cyclin-dependent kinase was inhibited. Anti-HP antibody also detected an immunoreactive band with a molecular weight of ~70 kDa in roscovitine-treated cells (Figure 3A, lane M/Ros). The nature of this band is unknown. These findings suggest that cyclin-dependent kinases phosphorylate the high-molecular-weight 4.1R in a mitosis-specific manner.

To ensure that the retarded 4.1R¹³⁵ isoform is due to phosphorylation, we performed an *in vitro* dephosphorylation of anti-HP immunoprecipitated 4.1R. We detected a 135-kDa and a retarded 135-kDa 4.1R using anti-HP antibody in the anti-HP immunoprecipitates from interphase and mitotic HeLa cells, respectively (Figure 3B, α -HP, lanes G1/S and M). Treatment of these immunoprecipitates with λ protein phosphatase eliminated the phosphorylated mitotic form of 4.1R (Figure 3B, λ PPase, lane M) as judged by the reduction of the shifted band to interphase mobility (Figure 3B, λ PPase, lane G1/S). These results suggest that the retarded 135-kDa 4.1R isoform in mitotic cell lysate is a phosphorylated form.

To determine whether the 135-kDa 4.1R isoform is specifically phosphorylated *in vivo*, we metabolically labeled G1/S and M phase HeLa cells with [³²P]orthophosphate and immunoprecipitated them with an anti-HP antibody. Auto-

Figure 3. 4.1R phosphorylation is mitosis-specific. (A) 4.1R mobility shift at the mitosis phase. Lysates of HeLa cells synchronized at the G0 phase (lane G0), G1/S phase (lane G1/S), S phase (lane S), G2 phase (lane G2), mitosis phase (lane M), and mitosis phase in the presence of roscovitine (lane M/Ros) were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose. The blot was probed with an anti-HP antibody. An unidentified ~70-kDa protein (block arrow) was detected in roscovitine-treated mitotic cell lysates. (B) In vitro dephosphorylation of 4.1R by λ protein phosphatase. Lysates of HeLa cells synchronized at the G1/S phase (lane G1/S) and mitosis phase (lane M) were subjected to immunoprecipitation using a rabbit preimmune IgG (Preim.) or an anti-4.1R antibody (α -HP). The resulting anti-HP immunoprecipitates were subjected to a dephosphorylation reaction in either the absence or presence of the λ protein phosphatase (λ PPase) and separated on 8% SDS-polyacrylamide gels. The proteins were identified by an immunoblot using an anti-HP antibody. (C) Mitosis-specific phosphorylation of 4.1R in vivo. Synchronized HeLa cells were incubated with [32 P]orthophosphate, harvested in either interphase (G1/S) or mitosis (M), and subjected to immunoprecipitation using a rabbit preimmune IgG (Preim.) or an anti-4.1R antibody (α -HP). The resulting immunoprecipitates were subjected to size fractionation on SDS-polyacrylamide gels, and the proteins were identified by immunoblotting using an anti-HP antibody (Immunoblot) or by autoradiographic exposure (Autoradiography).

radiography detected a 32 P-labeled 135-kDa 4.1R and a slightly retarded 135-kDa 4.1R in anti-HP immunoprecipitates from G1/S and M phase cells, respectively (Figure 3C, Autoradiography, α -HP, lanes G1/S and M, arrow). We detected no corresponding bands in the immunoprecipitates of preimmune IgG (Figure 3C, Autoradiography, Preim., lanes G1/S and M). We also detected several 32 P-labeled bands in anti-HP immunoprecipitates. The identities of these bands are unknown; they could represent 4.1R-associated proteins because our previous results (Mattagajasingh *et al.*, 1999) showed that 4.1R associates in a complex with mitotic-apparatus proteins during mitosis. Immunoblot analysis demonstrated that 4.1R from mitotic lysates also displayed a slightly retarded electrophoretic mobility relative to that derived from interphase cells (Figure 3C, Immu-

noblot, α -HP, lanes G1/S and M, arrow). These data demonstrate that the 135-kDa 4.1R isoform is a phosphoprotein that is hyperphosphorylated in the mitosis phase of the cell cycle.

4.1R Is Phosphorylated In Vitro by p34^{cdc2} Kinase

We next used in vitro phosphorylation assays to determine whether cdc2 kinase could phosphorylate 4.1R. Three cdc2 kinase phosphorylation sites (S/T-P-X-Z; where X is polar amino acid and Z is generally basic; Monero and Nurse, 1990), which closely conform to the consensus motif for phosphorylation by cdc2, are conserved among several species of 4.1R (human, mouse, rat, and dog; Figure 4A). These motifs are located within the HP (Thr⁶⁰, TPTH), the 16-kDa domain (Ser⁵⁵⁵, SPRP), and the junction of the SAB and the

Figure 4. 4.1R is phosphorylated at Thr⁶⁰ and Ser⁶⁷⁹ by purified cdc2 kinase. (A) Alignment of the deduced amino acid sequences of human (accession number AAD4222.1), mouse (accession number NP_906273), rat (accession number XP_232771), and dog (accession number AY553843) 4.1R. The aligned sequences located in the HP (amino acid 60–63), the 16-kDa domain (amino acid 555–558), and the SAB/CTD domain (amino acid 679–682) conform to the consensus motif (S/T-P-X-Z) for phosphorylation by cdc2 kinase. (B) In vitro phosphorylation of 4.1R by cdc2 kinase. Wild-type HP (HP-WT), wild-type 16 kDa (16 kDa-WT), wild-type SAB+CTD (SAB+CTD-WT), and their respective mutants HP^{T60A} (HP-Mut), 16 kDa^{S555A} (16 kDa-Mut), and SAB+CTD^{S679A} (SAB+CTD-Mut) were expressed as GST-tagged fusion proteins. Two micrograms of each fusion were incubated with purified cdc2/cyclin B for 30 min in the presence of [γ - 32 P]ATP and fractionated on an SDS-polyacrylamide gel. The phosphorylated fusion proteins were visualized by autoradiography. (C) Coomassie Blue-stained SDS-polyacrylamide gel illustrating the relative loading and size of the fusion proteins examined for cdc2 phosphorylation. The asterisk (*) indicates the degradation products of GST-16-kDa fusion.

Figure 5. Characterization of 4.1R Thr⁶⁰ phosphorylation in mitotic extracts, immunoprecipitates of tubulin and NuMA, and in the microtubule pellet of mitotic aster assembled *in vitro*. (A) Anti-HP immunoprecipitates from extracts of G1/S or M phase HeLa cells were analyzed by immunoblotting using anti-HP or anti-Thr⁶⁰ antibodies. (B) Immunoprecipitates from preimmune IgG, α -HP, α -tubulin, and α -NuMA prepared from mitotic HeLa extracts were immunoblotted with anti-HP or anti-Thr⁶⁰ antibodies. As a control, 25 μ g of mitotic cell lysate were also loaded. (C) Western blot analysis of immunodepleted mitotic extracts. Extracts were successively depleted three times with 30 μ g of anti-HP antibodies. The degree of 4.1R depletion was determined by the presence of 4.1R in protein A Sepharose-bound fractions as indicated by P1, P2, and P3; unbound fractions by S1, S2, and S3. For the control, 25 μ g of mitotic cell lysates were loaded. (D) 4.1R is required for the assembly of mitotic asters *in vitro*. Control mitotic (Control), preimmune-depleted (Pre-immune depleted), 4.1R-immunodepleted (α -HP depleted), and the anti-HP-immunodepleted and recombinant 4.1R¹³⁵-supplemented (Reconstituted) extracts were assembled under optimal conditions with taxol and ATP for 60 min at 30°C and processed for

indirect immunofluorescence using anti-tubulin and anti-NuMA antibodies. Bar, 5 μ m. (E) Recombinant 4.1R¹³⁵ is readily phosphorylated in mitotic extracts and incorporated into the insoluble microtubule fraction. Mitotic asters were assembled in the 4.1R-depleted mitotic extract with the addition of purified 4.1R¹³⁵ recombinant protein (at the same concentration as that of endogenous 4.1R) under optimal conditions. The assembled asters were separated into soluble (Sup) and insoluble (Pellet) fractions by centrifugation through a 50% sucrose cushion. Ten percent of the supernatant and the entire pellet were loaded. The recombinant 4.1R¹³⁵ (lane Input) was included as a control. These fractions were subjected to an immunoblot analysis using anti-HP (left panel) or anti-Thr⁶⁰ (right panel) antibodies.

CTD (Ser⁶⁷⁹, SPFR). We expressed the HP, the 16-kDa, and the SAB+CTD domains (as well as their mutated versions) as GST fusion proteins and incubated each GST-4.1R fusion with recombinant p34^{cdc2}/cyclin B kinase in the presence of [γ -³²P]ATP. The wild-type HP/GST and SAB+CTD/GST phosphorylated readily (Figure 4B, HP-WT, SAB+CTD-WT). However, mutation of Thr⁶⁰ to alanine completely abolished cdc2-mediated phosphorylation of HP/Mut (Figure 4B, HP-Mut). Similarly, mutation of Ser⁶⁷⁹ to alanine also abolished phosphorylation of the SAB+CTD domain (Figure 4B, SAB+CTD-Mut). Phosphorylation did not occur with the wild-type and mutated 16 kDa/GST (Figure 4B, 16 kDa-WT, 16 kDa-Mut) or the GST alone (Figure 4B, GST+cdc2). We normalized the amount of fusion protein in this experiment with Coomassie Blue staining (Figure 4C). These results suggest that Thr⁶⁰ and Ser⁶⁷⁹ are the primary sites for 4.1R phosphorylation by cdc2 kinase.

Phosphorylated 4.1R Associates with NuMA and Tubulin *In Vivo* as well as *In Vitro*

Having identified Thr⁶⁰ and Ser⁶⁷⁹ of 4.1R as the cdc2 kinase phosphorylation sites, we subsequently determined whether these two sites are phosphorylated in mitotic HeLa cells by using antibodies generated against synthetic phospho-Thr⁶⁰ or phospho-Ser⁶⁷⁹ peptides. The anti-Ser⁶⁷⁹ antibody appears to be only sequence-specific for 4.1R because it does not immunoreact with interphase and mitotic cell lysates after affinity chromatography on a phospho-Ser⁶⁷⁹ peptide column, followed by absorption with a nonphosphopeptide column. On the other hand, the affinity-purified anti-Thr⁶⁰ antibody specifically recognizes strong immunoreactive ~135- and ~175-kDa 4.1R bands from mitotic lysates, but

only a slightly reactive ~135-kDa band from interphase lysates. Preabsorption of anti-Thr⁶⁰ antibody with its antigen completely abolished the staining pattern (unpublished data), which suggests that the anti-Thr⁶⁰ antibody is specific for Thr⁶⁰-phosphorylated 4.1R. Thus, we further used anti-Thr⁶⁰ antibody in detecting Thr⁶⁰-phosphorylated 4.1R.

Immunoblotting, using anti-HP antibody on interphase and mitotic HeLa lysates, gave approximately the same intensity of immunoreactive bands with molecular masses of ~135 and ~175 kDa in interphase lysates (Figure 5A, α -HP, lane G1/S) and corresponding shifted bands in mitotic lysates (Figure 5A, α -HP, lane M). When a duplicate membrane was immunoblotted with anti-Thr⁶⁰ antibody, intense signals with the same molecular weight as that of mitotic 4.1R (~135 and ~175 kDa) probed with anti-HP antibody were easily detected in the mitotic cell lysates (Figure 5A, α -Thr⁶⁰, lane M). We also detected a light intensity of the anti-Thr⁶⁰ immunoreactive band with a molecular mass of ~135 kDa in the G1/S cell lysate (Figure 5A, α -Thr⁶⁰, lane G1/S). These results suggest that the major fraction of the high molecular weight of mitotic 4.1R and a minor fraction of the interphase 4.1R are phosphorylated at Thr⁶⁰.

We have previously (Mattagajasingh *et al.*, 1999) shown that a 135-kDa 4.1R isoform associates in a complex with NuMA, dynein, and dynactin in mitotic cells. 4.1R also interacts and partially colocalizes with NuMA (Mattagajasingh *et al.*, 1999) and tubulin (Huang *et al.*, 2004) in mitotic cells. Therefore, we determined whether the Thr⁶⁰-phosphorylated 4.1R isoforms associated with the mitotic apparatus organization complex by examining anti-HP, antitubulin, or anti-NuMA coimmunoprecipitated complexes with

the anti-Thr⁶⁰ antibody. Anti-HP antibody detected 4.1R isoforms, immunoreactive bands of major ~135-kDa doublets and minor ~175-kDa doublets, from the immunoprecipitates of 4.1R (Figure 5B, α -HP, lane α -HP). We also detected a lighter intensity of the anti-HP immunoreactive band with a molecular weight of ~135-kDa doublets in the immunoprecipitates of α -tubulin and α -NuMA (Figure 5B, α -HP, lanes α -tubulin and α -NuMA). Immunoblotting of a duplicated membrane using anti-Thr⁶⁰ antibody revealed 4.1R isoforms of the same molecular weight from the immunoprecipitates of α -HP, α -tubulin, and α -NuMA (Figure 5B, α -Thr⁶⁰, lanes α -HP, α -tubulin, α -NuMA). These results further suggest that Thr⁶⁰-phosphorylated 4.1R is associated in the mitotic complex with the mitotic apparatus-organization proteins NuMA and tubulin.

To determine the function of 4.1R¹³⁵ phosphorylation, we asked whether phosphorylated 4.1R¹³⁵ participated in the organization of microtubules into mitotic asters in a cell-free mitotic extract prepared from synchronized HeLa cells (Gaglio *et al.*, 1997). For this experiment, we used the anti-HP antibody to immunodeplete the endogenous 4.1R from the cell extracts. We also followed the phosphorylation status of the added recombinant 4.1R¹³⁵ in mitotic asters assembled *in vitro*. In three successive depletions before the assembly reaction, the extracts were immunodepleted with either 30 μ g of affinity-purified anti-HP or purified preimmune rabbit IgG. We used Western blotting to examine the degree to which 4.1R was depleted from the extracts (Figure 5C). Preimmune IgG did not immunodeplete any 4.1R from the cell lysate (unpublished data), whereas anti-HP antibodies efficiently depleted ~95% of 4.1R in the first depletion (Figure 5C, lane P1). The second depletion removed the remaining ~5% of 4.1R (Figure 5C, lane P2). 4.1R was completely absent from the final supernatant (Figure 5C, lane S-3). We then stimulated microtubule assembly in the mitotic extract by adding 10 μ M taxol and 2.5 mM ATP and incubated the reaction at 30°C for 60 min. We examined the presence of tubulin and NuMA in the assembled mitotic asters using their respective antibodies. Under these conditions, the microtubules polymerized and organized into radial arrays, with NuMA concentrated at the central core in control lysates (Figure 5D, Control; Gaglio *et al.*, 1997). The depletion of 4.1R inhibited formation of mitotic asters and yielded randomly arranged microtubules (Figure 5D, α -HP-depleted), whereas the preimmune depletion did not affect mitotic aster assembly (Figure 5D, Preimmune-depleted). Furthermore, when we added a recombinant 4.1R¹³⁵ isoform at a concentration equivalent to that of endogenous 4.1R, the mitotic aster assembly reconstituted in a 4.1R-immunodepleted extract (Figure 5D, Reconstituted). It is worth noting that under the standardized conditions as described in *Materials and Methods*, we consistently observed ~20–25 asters per field under an 100 $\times$ objective in either control or preimmune depleted mitotic extracts. We examined ~400 asters with approximately the same size and morphology in three independent assays. The reconstitution experiments using the recombinant 4.1R¹³⁵ restored the assembly efficiency as well as the morphology to that of the preimmune depleted extracts.

Microtubule-associated proteins are found in the insoluble components when mitotic asters are centrifuged through 50% sucrose cushions (Mack and Compton, 2001). We analyzed the phosphorylation state of 4.1R in the 50% sucrose pellet of the reconstitution assays. Anti-HP antibody detected the recombinant 4.1R¹³⁵ (Figure 5E, α -HP, lane Input). Interestingly, we also detected mobility-shifted bands with molecular masses of ~145 and 175 kDa in the supernatant

and the pellet of the 50% pellet in addition to the 135-kDa band (Figure 5E, α -HP, lanes Sup and Pellet). These results suggest that the recombinant 4.1R underwent posttranslational modification in the mitotic HeLa cell extracts. The ~145-kDa mobility-shifted bands were also recognized by the anti-Thr⁶⁰ antibody when we blotted a duplicated membrane (Figure 5E, α -Thr⁶⁰, lanes Sup and Pellet). The higher molecular mass ~175-kDa band did not immunoreact with the anti-Thr⁶⁰ antibody. The nature of this shifted band is unknown. These results suggest that recombinant 4.1R¹³⁵ is readily phosphorylated in HeLa mitotic extract at Thr⁶⁰, incorporated into the mitotic asters, and associated with mitotic microtubule proteins.

Mutation of p34^{cdc2} Phosphorylation Site Abolishes Localization of 4.1R¹³⁵ to the Mitotic Spindle

To determine the function of the cdc2 kinase phosphorylation of 4.1R, we examined the intracellular localization of wild-type and mutated 4.1R during mitosis. We constructed GFP fusions with either wild-type 4.1R¹³⁵ or mutated 4.1R^{135(T60A,S679A)}, in which both Thr⁶⁰ and Ser⁶⁷⁹ were mutated to alanine and subcloned into a pTRE vector. Each isoform was introduced into HeLa Tet-Off cells to generate double-stable cell lines. Because overexpression of 4.1R isoforms causes apoptosis of the transfected cells (Mattagajasingh *et al.*, 1999), we adjusted the level of GFP-4.1R expression, as measured by quantitative immunoblotting, to levels equivalent to that of endogenous 4.1R by adjusting the amount of doxycycline in medium.

We examined the intracellular localization of 4.1R in stably transfected mitotic cells by visualizing GFP in relation to NuMA localization. GFP/4.1R¹³⁵ localizes and concentrates at the mitotic spindle and spindle poles in addition to overall cytoplasmic distribution (Figure 6A, top panel, lane GFP-WT). The intense spindle and spindle-pole localization of GFP/4.1R¹³⁵ coincides with that of NuMA (Figure 6A, top panel, lane NuMA) as confirmed by the superimposition of these images (Figure 6A, top panel, lane Merged). Mutation of Thr⁶⁰ and Ser⁶⁷⁹ to alanine, however, impeded localization of the mutated GFP/4.1R^{135(T60A,S679A)} proteins in the mitotic spindle and spindle poles (Figure 6A, bottom panel, lane GFP-Mut). Careful focusing of the microscope through different focal planes indicated that GFP/4.1R^{135(T60A,S679A)} proteins were diffusely localized throughout the cytoplasm but were not concentrated at the mitotic apparatus, where NuMA resides (Figure 6A, bottom panel, lane NuMA). These results strongly suggest that the conserved phosphorylation sites, Thr⁶⁰ and Ser⁶⁷⁹, are critical to the targeting of the 4.1R protein onto the mitotic spindle.

Phosphorylation of 4.1R¹³⁵ Enhances Its Association with NuMA and Tubulin and Is Critical to Mitotic Aster Assembly *In Vitro*

Because the mutation of p34^{cdc2} phosphorylation-site abolishes localization of 4.1R¹³⁵ to the mitotic spindle, we further investigated the effect of phosphorylation on 4.1R's association with NuMA and tubulin as well as on mitotic aster formation. We used coimmunoprecipitation to examine whether phosphorylation of 4.1R is required for its association with NuMA and tubulin using HeLa cells expressing GFP-4.1R¹³⁵ and GFP-4.1R^{135(T60A,S679A)}. Attempts to enrich mitotic cells with nocodazole using the HeLa Tet-Off lines were unsuccessful because the cells underwent apoptosis for unknown reasons. We thus analyzed these associations in HeLa transiently transfected with 4.1R¹³⁵/GFP or 4.1R^{135(T60A,S679A)}/GFP.

the GFP-4.1R^{135(T60A,S679A)} (Mut-4X) were loaded onto lanes as indicated. For the control, 75 μ g of cell lysates were loaded. (C) Western blot analysis of immunodepleted mitotic extracts and the added recombinant 4.1R. Extracts were successively depleted three times with 30 μ g of anti-HP antibodies. The presence of 4.1R in protein A Sepharose-bound fractions are indicated by P1, P2, and P3; unbound fractions by S1, S2, and S3. Recombinant 4.1R¹³⁵ (lane WT) and 4.1R^{135(T60A,S679A)} (lane Mut) added in the immunodepleted extracts. For the control, 25 μ g of mitotic cell lysates were loaded. (D) Reconstitution of mitotic-aster formation in the 4.1R-depleted mitotic extract using the purified recombinant 4.1R¹³⁵ or 4.1R^{135(T60A,S679A)}. Mitotic asters were assembled in the 4.1R-depleted mitotic extract under optimal conditions with taxol and ATP at 30°C. At the initiation of microtubule polymerization, the purified recombinant 4.1R protein was added in amounts equivalent to that of endogenous 4.1R. The samples were then processed for indirect immunofluorescence with anti- α -tubulin (green) or anti-NuMA (red) antibodies. Bar, 5 μ m.

Transfection efficiencies were ~40% for both the wild-type and the mutated constructs as visualized under a fluorescent microscopy for the expression of GFP. Twelve hours after transfection, mitotic cells were enriched with the addition of 60 ng/ml nocodazole for 8 h and subjected to immunoprecipitation using anti-GFP, anti-NuMA, and antitubulin antibodies. The immunoprecipitates were then immunoblotted with an anti-GFP antibody. The anti-GFP antibody detected an abundant amount of ~170-kDa GFP-4.1R fusion in anti-GFP immunoprecipitates (Figure 6B, WT, lane α -GFP) and small amounts of GFP-4.1R in the anti-NuMA and antitubulin immunoprecipitates (Figure 6B, WT, lanes α -NuMA and α -tubulin). When a similar amount of immunoprecipitate from the GFP-4.1R^{135(T60A,S679A)} transfected cell lysates were analyzed (Figure 6B, Mut-1X), an intense GFP band was detected in anti-GFP immunoprecipitates (Figure 6B, Mut-1X, lane α -GFP). However, only a minuscule amount of GFP was detected in the anti-NuMA and antitubulin immunoprecipitates (Figure 6B, Mut-1X, lanes α -NuMA and α -tubulin). The association of the mutated 4.1R with NuMA and tubulin becomes more evident when four times as much of the anti-NuMA and antitubulin immunoprecipitates was examined (Figure 6B, Mut-5X, lanes α -NuMA and α -tubulin). Our *in vitro* binding experiments suggest that 4.1R interacts with NuMA through its CTD (Mattagajasingh *et al.*, 1999), whereas both the MBD and the CTD of 4.1R possess binding activity for tubulin (Huang *et al.*, 2004). Consistent with the *in vitro* binding results, these data suggest that the wild-type and mutated 4.1R¹³⁵ can associate with NuMA and tubulin in the same complex. However, the wild-type 4.1R¹³⁵ has a much higher binding affinity for NuMA and tubulin, whereas the mutated form is less efficient in its association with these proteins. Thus, the phosphorylation of 4.1R¹³⁵ enhances its binding affinity for NuMA and tubulin.

Figure 6. Effect of Thr60 and Ser679 mutations on the localization of 4.1R, association with NuMA and tubulin, and mitotic aster assemblies. (A) Analysis of the localization of the exogenously expressed GFP-4.1R¹³⁵ and GFP-4.1R^{135(T60A,S679A)} relative to that of NuMA in mitotic HeLa cells. HeLa Tet-Off cells stably expressing GFP-4.1R¹³⁵ or 4.1R^{135(T60A,S679A)} were cultured in the presence of 100 ng/ml doxycycline. Cells were fixed and double-stained for α -NuMA (NuMA) and nucleic acids (DNA). Top: the distribution of GFP-4.1R¹³⁵ (green) and NuMA (red); bottom: the localization of GFP-4.1R^{135(T60A,S679A)} (green) and NuMA (red). Bar, 10 μ m. (B) Coimmunoprecipitation of NuMA, tubulin, and exogenously expressed GFP-4.1R¹³⁵ or GFP-4.1R^{135(T60A,S679A)} in HeLa mitotic extracts. HeLa mitotic extracts were prepared from transiently transfected cells enriched with 60 ng/ml nocodazole and subjected to immunoprecipitation using anti-GFP, anti- α -tubulin mAb, and anti-NuMA mAb. The immunoprecipitates were immunoblotted with anti-GFP antibodies. One fifth of the α -NuMA and α -tubulin immunoprecipitates from the GFP-4.1R¹³⁵ (WT) and GFP-4.1R^{135(T60A,S679A)} (Mut-1X) and four fifth of the immunoprecipitates from

We further assessed whether mutated 4.1R can support aster assembly in lysates where endogenous 4.1R has been depleted. We immunodepleted 4.1R from the mitotic lysate, reconstituted the aster formation with recombinant 4.1R¹³⁵ or 4.1R^{135(T60A,S679A)} at a concentration equal to that of endogenous 4.1R, and examined the efficiency of aster formation. Western blotting verified the complete depletion of the endogenous 4.1R (Figure 6C, lane S3) and the addition of the recombinant 4.1R in the depleted lysates (Figure 6C, lanes WT and Mut). The addition of 4.1R¹³⁵ supported aster formation in which microtubules arranged in a radial array that contained NuMA at the central core (Figure 6D, WT). The addition of 4.1R^{135(T60A,S679A)} resulted in much smaller structures that were also composed of both NuMA and tubulin (Figure 6D, Mut). However, the structures had very little or no microtubule radial arrays extending outward from the central core. These results suggest that the phosphorylated 4.1R¹³⁵ is required for efficient mitotic aster assemblies *in vitro*.

Suppression of 4.1R by siRNA Causes Defective Metaphase and Multinuclei

To identify the functional activity of 4.1R in cell division, we suppressed 4.1R protein levels in HeLa cells using a mixture of four siRNA duplexes. The siRNA sequence for targeting 4.1R was from positions 700 to 718, 831 to 849, 1351 to 1369, and 1372 to 1390 relative to the first nucleotide of human 4.1R (accession number J03796.1). Immunoblot analysis showed that the level of endogenous 4.1R was reduced by more than 70% within 24 h after cells were transfected with duplexes directed at 4.1R, compared with control cells, which were transfected with the nonspecific GL2 duplex (Figure 7A).

We assayed the organization of the mitotic spindle, chromosome, and nucleus in the cells 24 and 48 h after transfection.

Figure 7. Suppression of 4.1R by siRNA results in defective metaphase and multinuclei cells. HeLa cells were analyzed at 24 and 48 h after transfection with either the control (GL2) or 4.1R-specific siRNA (siRNA). (A) Western blot of cell lysates from untreated (lane lysate), 4.1R siRNA-treated (lane 4.1R), and control GL2 siRNA-treated (lane GL2) HeLa cells harvested 24 h after transfection and probed with antibodies against 4.1R (α -HP) or α -actin (α -actin; for loading control). (B) Metaphase spindle appearance 24 and 48 h after transfection. Cells were fixed and double-stained for α -tubulin (Tubulin) and nucleic acids (DNA). Bar, 10 μ m. (C) Interphase cells 48 h after transfection with control GL2 (GL2) or 4.1R siRNA (siRNA) were fixed and double-stained for α -tubulin (Tubulin) and nucleic acids (DNA). Bar, 10 μ m. (D) Quantification of the observed abnormal metaphase defects, multipolar spindles, and multinucleated interphase. One hundred cells in metaphase from each of the three independent experiments were scored for normal and disorganized phenotype. For nuclei, 300 interphase cells from each of the three independent experiments were scored for normal and multinuclei phenotype.

tion, at which time one or two cell cycles had been completed with suppressed 4.1R levels. Indirect immunofluorescence microscopy using anti- α -tubulin antibody showed metaphase defects 24 h after transfection (Figure 7B). In control cells, α -tubulin labeling showed spindle microtubules that converged into two tightly focused spindle poles (Figure 7B, GL2). In contrast, although bipolar mitotic spindles also formed in 4.1R siRNA-treated cells, the spindle poles showed broader and poorly focused microtubules (Figure 7B, siRNA-24 h). These spindle poles are also associated with less well-condensed chromosomes. Although ~3–5% of the control cells exhibited disordered spindle poles, ~15–20% of the 4.1R siRNA-treated cells displayed defective spindle poles ($n = 100$ metaphase cells, three independent trials; Figure 7D, 24 h-metaphase defect). Forty-eight hours after transfection, multipolar mitotic spindles with disorganized chromosomes (Figure 7B, siRNA-48 h) accounted for ~20% of metaphase cells examined. Approximately 5% of the control GL2 duplex-transfected metaphase cells also exhibited multipolar mitotic defects. 4.1R siRNA-treated cells consistently displayed four to five times more defective metaphase cells than control cells ($n = 100$ metaphase cells, three independent trials; Figure 7D, 48-h multipolar spindle). However, ~75% of metaphase cells appeared to have normal spindles despite efficient 4.1R suppression. These multipolar mitoses appear to lead to an increasing number of cells with two or more nuclei (Figure 7C). We observed ~15–20% of 4.1R siRNA-treated cells displayed multinuclei, whereas only ~5% of GL2-treated cells showed multinuclei ($n = 300$, three independent trials; Figure 7D, 48-h multinucleated interphase). The combined results suggest that 4.1R is an important protein involved in the events of spindle formation that most likely occur before metaphase.

DISCUSSION

Nonerythroid cells express a diverse array of 4.1R isoforms with distinct functions. The most striking feature of 4.1R in nonerythroid cells is its cell cycle-dependent localization. In this study, we identified 4.1R¹³⁵ as the predominant isoform expressed in both interphase and mitotic HeLa cells. We also demonstrated that 4.1R is important in the organization of

the mitotic microtubule network through a molecular mechanism by which dynamic cellular relocalization of 4.1R is regulated by cdc2 kinase in a cell cycle-dependent manner.

In agreement with an earlier report (Parra *et al.*, 2003), we found that the expression of exons 1A, 1B, and 1C in HeLa cells is mutually exclusive. Our analysis of 4.1R mRNA showed that transcripts containing exons 1A or 1C are the predominant forms and exon 1B is not used readily in HeLa cells. Although the mechanism by which these first exons are expressed remains to be determined, the selective utilization of these exons seems to be tissue- or cell-type specific. Ramez *et al.*, (2003) reported cell-specific expression of the first exon in mouse kidney, in which the major renal 4.1R is a 105-kDa isoform containing exon 1A but lacking exons 1B and 1C. We (our unpublished data) have observed reciprocal expression of exons 1B and 1C in a wide variety of mouse tissues and cell lines. Clearly, coupled transcription and splicing regulation is added to the already complex 4.1R gene-regulation pathways.

4.1G, 4.1N, and 4.1B display ~70% sequence similarity within the conserved MBD, SAB, and CTD domains of 4.1R (Parra *et al.*, 1998; Walensky *et al.*, 1999; Yamakawa *et al.*, 1999; Ye *et al.*, 1999; Parra *et al.*, 2000). Given this multitude of similarities, it has become a point of significant interest to characterize potential functional differences among these four proteins. In this study, we used anti-HP antibody that allowed us to distinguish 4.1R from other 4.1 gene families in order to trace 4.1R-specific polypeptides during the cell cycle. Using this 4.1R-specific antibody and 4.1R-specific mini-cDNA libraries, we identified a 4.1R¹³⁵ isoform as the predominant form in HeLa interphase cells. This isoform has been identified in erythroblasts (Gascard *et al.*, 1998) and in a wide variety of nucleated nonerythroid cells (Tang *et al.*, 1988, 1990; DeCarcer *et al.*, 1995; Krauss *et al.*, 1997b; Kontogianni-Konstantopoulou *et al.*, 2000); these data suggest that 4.1R¹³⁵ may exhibit a common function in different cell types. In mitotic cells, 4.1R¹³⁵ isoform is phosphorylated at Thr⁶⁰ and Ser⁶⁷⁹. These two amino acids appear to be critical in directing 4.1R onto the mitotic spindle and in the association with NuMA and tubulin. Each amino acid resides in clusters of at least 18 consecutive amino acids that are perfectly conserved among human, mouse, rat, and dog 4.1R, which signifies their importance in regulatory functions. The

fact that phosphorylated 4.1R¹³⁵ associates with NuMA and tubulin in mitotic HeLa cells in vivo and in mitotic aster assembled in vitro suggest that phosphorylation controls 4.1R's interaction with mitotic apparatus proteins. The ready phosphorylation of a recombinant 4.1R¹³⁵ and its reconstitution of mitotic-aster assembly in 4.1R-immunodepleted extracts further supports this hypothesis.

Our results indicate that nonphosphorylated 4.1R forms localize in the interphase nucleus and in the cytoplasmic network, and their phosphorylated forms distribute to the spindle and spindle poles of mitotic cells. Key transitions in the cell cycle are controlled by cyclin-dependent kinases. Complexes of p34^{cdc2} with B-type cyclins are responsible for promoting the entry of cells into mitosis (Hunt, 1991; Nigg, 1995). Mitotic activation of cdc2 kinase modifies multiple proteins that are involved in different processes of cell division (Liao *et al.*, 1994; Blangy *et al.*, 1995; Compton and Luo, 1995; Nikolakaki *et al.*, 1997). Mitotic disassembly of the nuclear lamina results from direct phosphorylation of lamins by cdc2 kinase (Peter *et al.*, 1990). Phosphorylation by cdc2 kinase regulates binding of kinesin-related motor HsEg5 to the dynactin subunit p150^{Glued} (Blangy *et al.*, 1995); this complex may play a role in mediating the association of cytoplasmic dynein and HsEg5 with the mitotic spindle apparatus. Cdc2 kinase phosphorylation regulates the association of NuMA with the mitotic spindle (Compton and Luo, 1995). Although direct evidence is still lacking, cdc2 kinase may serve as a master switch for regulating the localization of 4.1R during mitosis. Thus, it is conceivable that the phosphorylated 4.1R¹³⁵ isoform is localized in distinct intracellular sites with respect to the unphosphorylated forms. Changes in the localization of 4.1R may serve as an effective mechanism for defining its binding partners in a temporally and spatially dependent manner. Conversely, 4.1R phosphorylation may alter its affinity for binding partners.

The biological significance of 4.1R in cell division is demonstrated by siRNA-induced suppression of 4.1R expression, which results in poorly focused spindle poles in mitotic cells. 4.1R also has been shown to be a crucial factor for assembly and maintenance of mitotic spindles in metaphase arrested *Xenopus* egg extracts (Krauss *et al.*, 2004). However, in our study a significant fraction of 4.1R-depleted cells eventually progressed to anaphase and cytokinesis. Cell division remains normal in 4.1R knockout mice (Shi *et al.*, 1999) despite the absence of 4.1R. It is worth noting that 4.1G expresses in wide variety of tissues and cell types, whereas 4.1N and B express in selected cell types. It is possible that the highly homologous 4.1 proteins 4.1G, 4.1B, and 4.1N may compensate for 4.1R loss in both 4.1R siRNA-suppressed cells and 4.1R knockout mice. This idea is supported by an immunofluorescent staining and an in vitro binding assay (Delhommeau *et al.*, 2002), showing that 4.1G also colocalizes with NuMA at the spindle poles of mitotic cells as well as interacts with NuMA in vitro. Whether 4.1G functions the same as 4.1R in cell division remains to be determined. Exactly how 4.1R contributes to spindle organization is unknown. Additional work will establish the precise role of the cytoskeletal protein 4.1R in the organization of the spindle and spindle poles. Nevertheless, our studies strongly suggest that 4.1R may be pivotal to the structural organization and maintenance of focused microtubule arrays. Our findings clearly indicate that cdc2 kinase-phosphorylated 4.1R isoforms are crucial to these processes.

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