A Nonerythroid Isoform of Protein 4.1R Interacts with the Nuclear Mitotic Apparatus (NuMA) Protein

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Abstract. Red blood cell protein 4.1 (4.1R) is an 80-kD erythrocyte phosphoprotein that stabilizes the spectrin/actin cytoskeleton. In nonerythroid cells, multiple 4.1R isoforms arise from a single gene by alternative splicing and predominantly code for a 135-kD isoform. This isoform contains a 209 amino acid extension at its NH_2 terminus (head piece; HP). Immunoreactive epitopes specific for HP have been detected within the cell nucleus, nuclear matrix, centrosomes, and parts of the mitotic apparatus in dividing cells. Using a yeast two-hybrid system, in vitro binding assays, coimmunolocalization, and coimmunoprecipitation studies, we show that a 135-kD 4.1R isoform specifically interacts with the nuclear mitotic apparatus (NuMA) protein. NuMA and 4.1R partially colocalize in the interphase nucleus of MDCK cells and redistribute to the spindle poles early in mitosis. Protein 4.1R associates with NuMA in the interphase nucleus and forms a complex with spindle pole organizing proteins, NuMA, dynein, and dynactin during cell division. Overexpression of a 135-kD isoform of 4.1R alters the normal distribution of NuMA in the interphase nucleus. The minimal sequence sufficient for this interaction has been mapped to the amino acids encoded by exons 20 and 21 of 4.1R and residues 1788–1810 of NuMA. Our results not only suggest that 4.1R could, possibly, play an important role in organizing the nuclear architecture, mitotic spindle, and spindle poles, but also could define a novel role for its 22–24-kD domain.

Key words: protein 4.1R • NuMA • dynein • dynactin • mitotic spindle

RED blood cell protein 4.1 (4.1R)[1] was identified as an 80-kD cytoskeletal protein in red cells (Ungewickell et al., 1979). It is critical for the organization and maintenance of the spectrin/actin cytoskeleton, and for the attachment of the cytoskeleton to the cell membrane through interactions with integral membrane proteins such as glycophorin C, and Band 3 (Tyler et al., 1979; Anderson et al., 1984; Pasternack et al., 1984). Abnormalities in 4.1R are associated with congenital red cell defects leading to membrane fragmentation and hereditary elliptocytosis. It is well established that the 80-kD 4.1R of mature red cells is only one member of a large family of 4.1R isoforms that arise from a single gene by alternative mRNA splicing (Tang et al., 1990; Conboy et al., 1991), usage of at least two translation initiation sites (Huang et al., 1992; Chasis et al., 1996), and posttranslational modifications (Subrahmanyam et al., 1991). Recently three new 4.1-like genes have been identified that share strong homology with the 30-, 10-, and 22–24-kD domains of 4.1R. One of them is generally expressed throughout the body (4.1G; Parra et al., 1998), another one is expressed in central and peripheral neurons (4.1N; Walensky, L.D., S. Blackshaw, J.G. Conboy, N. Mohandas, and S.H. Snyder. 1997. *Soc. Neurosci.* 23:1674a), and a third one is expressed in brain (4.1B; Parra, M., P. Gascard, L.D. Walensky, S.H. Snyder, N. Mohandas, and J.G. Conboy. 1997. *Mol. Biol. Cell.* [Suppl.] 8:177a).

The major functions of erythrocyte 4.1R have been well described. The 30-kD NH_2-terminal domain of erythrocyte 4.1R interacts with glycophorin C, calmodulin, p55 (Tanaka et al., 1991; Hemming et al., 1994; Marfatia et al., 1995), and band 3 (Jons and Drenckhahn, 1992). The 10-kD

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1. *Abbreviations used in this paper:* Ab, polyclonal antibody; 4.1R, red blood cell protein 4.1; Gal4-AD, Gal4 activation domain; Gal4-BD, Gal4 DNA-binding domain; HP, head piece or the NH_2-terminal extension of 4.1R; IP, immunoprecipitation buffer; NuMA, nuclear mitotic apparatus protein.

domain contains binding sites for spectrin and actin complexes (Correas et al., 1986; Discher et al., 1993; Horne et al., 1993). Purified 4.1R also interacts specifically with tubulin (Correas and Avila, 1988) and myosin (Pasternack and Racusen, 1989).

Isoforms of 4.1R are widely expressed in many tissues and are phylogenetically conserved. They are heterogeneous with regard to molecular mass, abundance, and cellular localization (Cohen et al., 1982; Granger and Lazarides, 1984, 1985; Anderson et al., 1988). Western blots of many types of mammalian and avian cells revealed 4.1R immunoreactive epitopes in protein species ranging from 30 to 210 kD (Granger and Lazarides, 1984; Anderson et al., 1988) in nucleated erythroid and nonerythroid cells. In contrast to the strict peripheral localization of 4.1R in mature red cells, 4.1R isoforms in nucleated cells are concentrated at points of cell–cell or cell–matrix contact, along stress fibers, on cytoplasmic matrixlike structures (Cohen et al., 1982; Lue et al., 1994), and perinuclear regions such as centrosomal and Golgi structures (Leto et al., 1986; Chasis et al., 1993). Several groups have also documented the presence of 4.1R isoforms in centrioles and the nucleus (Madri et al., 1988; De Carcer et al., 1995; Krauss et al., 1997a,b). 4.1R isoforms have also been observed as a component of the nuclear matrix of mammalian cells (De Carcer et al., 1995; Krauss et al., 1997b). Transcription-dependent redistribution of nuclear 4.1R to SC35-enriched nuclear domains suggests that 4.1R, in the nucleus, may be involved in splicing processes (Lallena and Correas, 1997). Also isoforms of 4.1R in the nucleus have been postulated to be significant contributors to nuclear architecture and serve as structural elements (Krauss et al., 1997b).

Our earlier observation (Marchesi, V.T., S. Huang, T.K. Tang, and E.J. Benz, Jr. 1990. *Blood.* 76:12a) and those of Krauss et al. (1997b) showed that 4.1R is located in the nucleus during interphase and it rapidly redistributes to the developing spindle poles when the nuclear envelope dissembles in prometaphase, a characteristic of nuclear matrix proteins that leave the nucleus during interphase/mitosis transition to become structural components of the mitotic apparatus, seen both in mammalian cells (for review see He et al., 1995) and *Drosophila* (Saunders et al., 1997). The multiplicity of 4.1R isoforms combined with the diversity of possible protein–protein interactions suggests that individual 4.1R isoforms may have specific and discrete functions. However, little is known about the cytochemistry, biochemical functions, or physiologic importance of 4.1R isoforms other than in red cells.

To understand the function of 4.1R isoform(s) in nonerythroid cells, we searched for proteins that interact with the 135-kD 4.1R. This isoform arises from alternative mRNA splicing resulting in the addition of 209 amino acids to the NH_2-terminal end of erythrocyte 4.1R (HP; Tang et al., 1990). We performed a yeast two-hybrid screen of a human brain cDNA library using 135-kD isoform of 4.1R as a bait and isolated several clones encoding the COOH-terminal domain of nuclear mitotic apparatus (NuMA) protein, a protein known to augment organization and stabilization of the mitotic spindle. NuMA was suggested to stabilize the parallel arrays of microtubules that emerge from the centrosomes early in mitosis (Yang and Snyder, 1992). During the transition from mitosis to interphase, NuMA may have a role in stabilizing the tethering of chromosomes to each spindle pole and/or provide structural support to the nucleus during the complex reassembly process (Kallajoki et al., 1991; Compton and Cleveland, 1993).

We report here that a 135-kD 4.1R isoform associates with NuMA in vivo and partially colocalizes at different stages of the cell cycle. This isoform also associates with NuMA, cytoplasmic dynein, and dynactin during cell division. Furthermore, its overexpression leads to alteration in the intracellular localization of NuMA in interphase. The later proteins are involved in tethering the microtubules at the spindle poles and are essential for mitotic spindle pole stabilization. Therefore, our results imply strongly that a nonerythroid 4.1R isoform may have a role in cell division.

Materials and Methods

Antibody Production

Antiserum to synthetic peptides of segments of 10 kD (MESVPEPRPSEWDK), 22–24 kD (GVLLTAQTITSETPSSTTTTKITKC, exon 19) domain, or to the recombinant HP of 4.1R (anti-HP 4.1) was produced. Anti-synthetic peptide antibodies were prepared and purified as described earlier (Leto et al., 1986). The DNA fragment containing the coding sequence for the 209 amino acids of HP of 4.1R was fused in frame to GST by using pGEX-2T vector, expressed in *Escherichia coli*, and purified on glutathione-Sepharose 4B beads according to the manufacturer's recommendations (Pharmacia Biotech Inc.). Aliquots of GST-HP 4.1 fusion protein were mixed with complete Freund's adjuvant and injected subcutaneously into New Zealand White male rabbits. 4 wk later the rabbits were given a booster dose of incomplete adjuvant. Sera were collected by bleeding through ear vein and were cleared by centrifugation. The sera were purified on immunoaffinity columns using resins covalently cross-linked to the fusion peptide used for immunization. The purified antibodies were eluted from the columns with 0.1 N acetic acid, neutralized, and dialyzed against 0.1 M borate buffer, pH 8.0.

Anti-NuMA antibodies (raised in rabbit) used in this study were previously characterized (Yang and Snyder, 1992). In some experiments, an anti-NuMA mAb (Oncogene) was also used. The anti–Na^+-K^+-ATPase α1 polyclonal antibody (Ab) used was described previously (Zahler et al., 1993). Antidynein mAb (intermediate chain, clone 70.1), anti-actin mAb, antispectrin (α and β) mAb, and anti–γ-tubulin Ab were purchased from Sigma Chemical Co. Anti-$p150^{glued}$ mAb (the largest subunit of dynactin) was purchased from Transduction Laboratories. Antipericentrin Ab and anticalmodulin mAb were purchased from BAbCO and Zymed Laboratories, Inc., respectively.

Plasmid Construction

cDNAs were generated by restriction digestion of 4.1R cDNAs (Tang et al., 1988) or by PCR using an amplification kit (Promega Corp.) and a thermocycler (PCR System 480; Perkin-Elmer Corp.). For the full-length (135-kD) 4.1R construct, the SmaI and BglII fragment from pTM-Full was subcloned into the SmaI and BamHI site of pAS2-1. For subcloning the 80-kD 4.1R or its different domains, DNA sequences were PCR amplified from 135 kD/PTM-Full or 80 kD/pEry-1,2,3 (Tang et al., 1988) using custom oligonucleotide primers (Genosys Biotechnologies) designed with SalI and PstI sites at their 5′ or 3′ ends, respectively. The primers designed for generation of 4.1R domains (see Fig. 1 A and GenBank/EMBL/DDBJ under accession number J03796) were as follows: Hp/pAS2-1 (47–69/651–673), 30 kD/pAS2-1 (674–696/1539–1461), 16 kD/pAS2-1 (1462–1484/1762–1784), 10 kD/pAS2-1 (1785–1807/1900–1922), and 22–24 kD/pAS2-1 (1923–1942/2354–2374). The amplified cDNAs were digested with SalI and PstI, purified, and cloned into SalI and PstI sites of Gal4 DNA-binding domain vectors, pGBT9 or pAS2-1, by standard methods.

Different NuMA constructs containing the NuMA cDNA corresponding to NH_2-terminal domain (308–878), coiled-coil domain (877–5335), or COOH-terminal domain (5356–6720) were cloned in frame with Gal4 transactivation domain in vector pACTII. The 135-kD/GST fusion construct was generated by subcloning the EcoRI and SalI insert of 135 kD/pAS2-1 into EcoRI and SalI sites of pGEX-6P1 (Pharmacia Biotech Inc.).

For 80 kD/GST and its different domains in GST vector pGEX-6P1, custom primer sets with the same sequences used for Gal4 DNA-binding domain (Gal4-BD) constructs, except with EcoRI or SalI at their 5′ or 3′ ends, were used to amplify the corresponding cDNA sequences, digested with EcoRI and SalI, and were subcloned in frame into pGEX-6P1. The 135^{++}kD/GFP 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 in frame into KpnI and BamHI sites of pEGFP-C1 (CLONTECH Laboratories). NuMA/TOPO constructs containing sequences corresponding to 5356–6561 (NuMA1/TOPO), 5356–5929 (NuMA2/TOPO), or 5752–6561 (NuMA3/TOPO) of NuMA (available from GenBank/EMBL/DDBJ under accession number Z11584) were amplified with addition of the Kozak consensus sequence and the translation initiation site (GCCACCATG) incorporated into the 5′ end and a stop codon at the 3′ end. The amplified fragments were cloned into pCR®-Blunt II-TOPO vector (Invitrogen Corp.). To ensure correctness of the reading frame, the 5′-junction of each construct was sequenced using a sequencing kit (sequenase v. 2.0; Amersham Corp.).

Deletion constructs representing different exons of the 22–24-kD domain of 4.1R were derived from 22–24 kD/pAS2-1 by using custom oligonucleotide primers as above, and were cloned into SalI and PstI sites of pGBT9. The primers (see Fig. 3 A and GenBank/EMBL/DDBJ under accession number J03796) used were as follows: exons 17–21/pGBT9 (1923–1942/2354–2374), exon 18–21/pGBT9 (1963–1981/2354–2374), exon 18–20/pGBT9 (1963–1981/2275–2295), exon 18–19/pGBT9 (1963–1981/2175–2194), exon 19/pGBT9 (2093–2112/2175–2194), exon 20/pGBT9 (2195–2217/2275–2295), exon 21 (2276–2298/2354–2374), and exon 20–21 (2195–2217/2354–2374). The construct exon 19–21/pGBT9 was made by a two-step PCR using the primer set (2093–2112/2164–2194 and 2276–2285) and (2185–2194 and 2276–2296/2354–2374) for the first round of amplification. The primary PCR products were annealed and the second round of PCR was done with the 5′and 3′ primers (2093–2112/2354–2374). The constructs exon 21 (amino acids 762–775)/pGBT9 and exon 21 (amino acids 745–761)/pAS2-1 were derived from 22–24 kD/pAS2-1 by using the SmaI and StyI sites, respectively. Different deletion constructs of NuMA were derived from NuMA 1476–2115/pACT2 or NuMA 1697–2115/pACT2 (see Fig. 1 B and GenBank/EMBL/DDBJ under accession number Z11584). The plasmids were digested with BglII, the fragment of interest was purified, digested with the available restriction enzyme at the 3′ site of interest, and blunt-ended. Afterwards, the cDNA fragments were digested with EcoRI, purified, and cloned into pACT2 at the EcoRI and blunt-ended XhoI sites. The NuMA 1788–1832/pACT2 construct was derived from NuMA 1697–2115/pACT2 using custom oligonucleotide primers (5621–5647 and 5736–5758, these data are available from GenBank/EMBL/DDBJ under accession number Z11584) designed with EcoRI and XhoI site at their 5′ or 3′ ends, respectively, as above. The authenticity of all deletions was confirmed by dideoxy sequencing in both directions.

Yeast Two-Hybrid Assay

The Gal4-based MATCHMAKER two-hybrid system II of CLONTECH Laboratories, Inc. was followed for the yeast two-hybrid assays. Plasmid vectors, pAS2-1 or pGBT9, and pACT2, encoding the Gal4-BD and Gal4-activating domain (Gal4-AD), respectively, were used to express hybrid proteins. To screen for proteins that interact with 4.1R in yeast two-hybrid system, a human brain cDNA library in Gal4-AD vector pACT2 was screened using either the full-length 4.1R (135-kD) or the 22–24 kD of 4.1R cloned into Gal4-BD vector (see plasmid construction section for details) as the bait. Positive clones were tested further for specificity by cotransformation into Y190 either with 4.1R 135 kD/pAS2-1, 22–24 kD/pAS2-1, or with pAS2-1 alone. DNA from positive clones were isolated, the Gal4-AD plasmids were recovered in bacteria strain HB 101, and sequenced by dideoxy method as above. For domain mapping, plasmids carrying respective inserts fused to Gal4-BD or Gal4-AD were cotransformed into yeast, and were assayed for β-galactosidase activity on nitrocellulose filters as described in CLONTECH Laboratories' manual.

In Vitro Binding Assay

The in vitro transcription/translation of NuMA/TOPO was performed using the TNT® coupled (reticulocyte lysate system; Promega Corp.) in the presence of [^{35}S]methionine to radiolabel newly synthesized proteins. Equal amounts of the labeled NuMA proteins were incubated with affinity-purified GST/4.1R fusion proteins coupled to glutathione-Sepharose beads for 1 h at 4°C in binding buffer (50 mM potassium phosphate, pH 7.3, 150 mM NaCl, 2.7 mM KCl, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml antipain, 12.5 μg/ml chymostatin, 12 μg/ml pepstatin, 130 μg/ml ϵ-amino caproic acid, 200 μg/ml *p*-NH_2-benzamidine, and 1 mM PMSF). After incubation, the immobilized NuMA-4.1R complex was washed five times with binding buffer in the presence of 1% Triton X-100, once with the binding buffer plus 1% Triton X-100, 2 M urea and 100 mM glycine, and once with the binding buffer. The bound protein complex was subjected to analysis on a 15% or 18% SDS-PAGE, treated with Enlightning (NEN Life Science), and visualized by fluorography.

Cell Culture, Transfection, and Synchronization

HeLa (ATCC CCL 2, human cervix epitheloid carcinoma) and MDCK (ATCC CRL 1772, Madin-Darby canine kidney) cells were grown in DME supplemented with 10% heat-inactivated FBS (GIBCO BRL). The cDNA encoding the full-length 4.1R (80 kD 4.1R [Baklouti et al., 1997] containing HP without exon 3) in pEGFP-C1 vector (135^{++}kD/GFP) or the vector pEGFP-C1 was transiently transfected into HeLa cells using LipofectAmine reagent following the manufacturer's procedure (GIBCO BRL). The 135^{++}kD/GFP cDNA contained all of the known 4.1R exons except exon 3 and the tissue-specific exons 14, 15, 17a/a′, and 17b. Cells in a 100-mm^2 dish were transfected for 16 h, the GFP- or 135^{++}kD/GFP–transfected cells were sorted using a FACStar® cell sorter to avoid the untransfected cell population overgrowth, and the transfected cells were collected. The sorted cells were plated on poly-D-lysine–coated coverslips and the samples were taken daily for 5 d, fixed, and processed for immunofluorescence staining. The transfected cells were immunofluorescence-stained using Texas red–conjugated anti–mouse IgG, anti-NuMA mAb, and TO-PRO-3 (for DNA staining), and were examined for the presence of 135^{++}kD/GFP fusion protein, Texas red, and Cy 5 fluorescence in individual cells.

Highly enriched mitotic HeLa cells were prepared by synchronization of the cells at G1/S boundary of the cell cycle by double thymidine block (O'Conner and Jackman, 1995). Cells were grown in complete medium for 4 h and the mitotic population was enriched by addition of 0.25 μg/ml of nocodazole for 6 h. Afterwards, cells were washed twice with complete medium, allowed to grow for 90 min in complete medium, and were collected by mitotic shake-off.

Subcellular Fractionation and Western Blotting

Expression of 4.1R and NuMA at protein level in MDCK cells was documented by Western blotting. Cytoplasmic-, nuclear-, and nuclear matrix-protein fractions were prepared as described (Mattagajasingh and Misra, 1996). The protein contents were determined using a protein determination kit (Pierce Chemical Co.). 20 μg of protein from each fraction was analyzed on 8–12% SDS-polyacrylamide gels (National Diagnostics Inc.). NuMA and 4.1R were detected by Western blotting using 1:500 dilution of anti-HP 4.1 or anti-NuMA antibodies as described earlier (Mattagajasingh and Misra, 1996) except that HRP conjugated goat anti–rabbit IgG and an ECL detection kit (Amersham Corp.) were used.

Coimmunoprecipitation and Immunoblotting

Coimmunoprecipitation of 4.1R and NuMA was performed using MDCK nuclear extracts. Nuclei were purified as described (Mattagajasingh and Misra, 1996), resuspended in coimmunoprecipitation (IP) buffer (25 mM Tris-HCl, 100 mM NaCl, 1 mg/ml BSA, 0.2 mM EDTA, 5 mM iodoacetamide, 0.05%[wt/vol] SDS, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml antipain, 12.5 μg/ml chymostatin, 12 μg/ml pepstatin, 130 μg/ml ϵ-amino caproic acid, 200 μg/ml *p*-amino-benzamidine, and 1 mM PMSF, pH 7.5), and given 20 strokes in a tight-fitting glass homogenizer. The nuclear homogenate was centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatant was used for coimmunoprecipitation. Preimmune serum (rabbit) equivalent to 30 μg IgG was added to 1 mg of nuclear proteins and incubated for 2 h at 4°C, followed by the addition of 200 μl of a 50% suspension of protein A–Sepharose 6 MB (Pharmacia) in IP buffer for 2 h at 4°C. The supernatant was collected by centrifugation at 5,000 *g* for 5 min at 4°C and equally split into five tubes. Preimmune serum, affinity-purified anti-HP 4.1 Ab, anti-NuMA mAb, mouse IgG, or anti-p53 mAb containing 4 μg IgG were added to different tubes and incubated for 2 h at 4°C. Protein A–Sepharose 6 MB (100 μl of a 50% suspension) was added and incubated as above.

For immunoprecipitation using mitotic HeLa extracts, cells were homogenized as above in IP buffer containing 170 mM NaCl, 2% Nonidet P-40, 0.1% (wt/vol) SDS, and 5 mM iodoacetamide. After centrifugation at 10,000 *g* for 10 min at 4°C, the supernatant was used for immunoprecip-

itation using 4 μg each of anti-NuMA mAb, anti-HP 4.1 Ab, antidynein mAb, anti-p150^{glued} mAb, anti-p53 mAb (an irrelevant antibody), mouse IgG or preimmune serum (rabbit), as above. After incubation with protein A–Sepharose, the samples were centrifuged for five min at 5,000 *g* at 4°C, and washed 5–10 times in 900 μl of IP buffer containing 1% Nonidet P-40 and 5 mM iodoacetamide. The samples were resuspended in 60 μl of SDS sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% [wt/vol] SDS, 5% 2-mercaptoethanol, and 10 μg/ml bromophenol blue, pH 6.8) and heated at 98°C for 5 min. The samples were centrifuged at 10,000 *g* for 10 min at 4°C and the supernatants were fractionated on 7–10% SDS-polyacrylamide gels. Transfer of proteins to nitrocellulose membrane and immunodetection of 4.1R or NuMA were carried out as described above. Some chemiluminograms were scanned using the Adobe Photoshop software (Adobe Systems, Inc.) and the protein bands of interest were quantitated by using the NIH Image software for the Apple Macintosh computer. In some cases, the gels were stained with the GelCode® Blue Reagent or the GelCode® SilverSNAP™ (Pierce Chemical Co.).

Fluorescence and Confocal Microscopy

MDCK or HeLa cells were grown on poly-D-lysine–coated glass coverslips. Coverslips were washed twice with PBS and immersed in a microtubule stabilizing buffer (4 M glycerol, 100 mM Pipes, pH 6.9, 1 mM EGTA, and 5 mM $MgCl_2$) for 2 min at room temperature and fixed in 2% paraformaldehyde, 0.4% glutaraldehyde, 90 mM Pipes, pH 6.8, 1 mM EGTA, and 5 mM $MgCl_2$ for 5 min at room temperature. Cells were washed three times with TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% albumin) and permeabilized with TBS containing 0.5% Triton X-100 for 5 min. Subsequently, the cells were washed three times with TBS containing 10 mM glycine. Cells were blocked with TBS containing 10% normal goat serum for 30 min at room temperature.

For single staining, cells were either incubated with anti-HP 4.1 antibody (diluted 1:50) or anti-NuMA antibody (diluted 1:100) in TBS for 1 h at room temperature. Cells were washed in 10 mM glycine/TBS and incubated with goat anti–rabbit IgG conjugated to FITC (for anti-HP 4.1) or donkey anti–rabbit IgG conjugated to Texas red (for NuMA) for 1 h at room temperature followed by four washes in 10 mM glycine/TBS. When double staining was desired, after the first set of staining, cells were blocked with TBS containing 10 μg/ml goat anti–rabbit Fab fragment followed by four washes in 10 mM glycine/TBS to reduce the nonspecific recognition by the second set of antibodies. Incubation with the second set of antibodies was the same as the first set of antibodies. Finally, the coverslips were mounted in mounting buffer (100 mM *n*-propyl gallate, 50% glycerol in PBS, pH 7.4) and the samples were viewed with a Nikon microphot-FXA or with a laser scanning confocal system (MRC 600; Bio-Rad Laboratories) coupled to a Zeiss Axiophot microscope through a 60× oil immersion objective. Images were processed using Photoshop software (Adobe Systems, Inc.) and photographed from the monitor screen.

Results

4.1R and NuMA Interact in the Yeast Two-Hybrid Assays

To understand the function of a predominant nonerythroid 4.1R isoform (135 kD), we detected its binding partners by using a yeast two-hybrid system. Using the fusion protein Gal4-BD/135 kD 4.1R (Fig. 1. A) as a bait in pAS2-1 and a human brain cDNA library fused to Gal4-AD in pACT2, we screened ~8 × 10^6 transformants. 79 positive clones were obtained, out of which 35 clones contained sequences that when translated would correspond to either amino acid residues 1476–2115 or 1697–2115 of NuMA (Fig. 1 B; these data are available from GenBank/EMBL/DDBJ under the accession number Z11584). When Gal4-BD/22–24 kD 4.1R was used as a bait (Fig. 1 A), some of the positive clones obtained also corresponded to the residues 1697–2115 of NuMA (these data are available from GenBank/EMBL/DDBJ under accession number Z11584). This interaction of 4.1R and NuMA in the two-hybrid assays seemed to be specific, because neither the Gal4-BD 4.1R hybrid interacted with the unfused Gal4-AD, nor did the Gal4-AD–NuMA hybrid (clones obtained from the library screening) interact with the unfused Gal4-BD (data not shown).

Three NuMA isoforms of 230, 195, and 194 kD have been reported. These isoforms arise by alternative splicing from a common precursor of a single NuMA gene but differ at their COOH termini (Tang et al., 1994). The NuMA protein has three different domains (Maekawa and Kuriyama, 1993). The NH_2- and COOH-terminal's globular domains correspond to residues 1–207 and 1729–2115, respectively. The middle α-helical coiled-coil domain comprises residues 208–1728. The positive clones obtained in the two-hybrid assays corresponded to the COOH-terminal end of the coiled-coil domain and the COOH-terminal globular domain. This could have derived from the 230-kD NuMA isoform because a part of these sequences was not detected in 195-kD and 194-kD isoforms of NuMA. In the 230-kD NuMA isoform, residues 1972–2007 were shown to contain a nuclear localization signal; the residues 1538–2115 are necessary and sufficient for its spindle association. This isoform of NuMA is present in interphase nuclei and has been shown to concentrate at the polar regions of the spindle apparatus in mitotic cells (Tang et al., 1994).

Protein 4.1R and NuMA Interact through their COOH-terminal Domains

To identify the domains of 4.1R and NuMA responsible for their interaction, we expressed different domains of

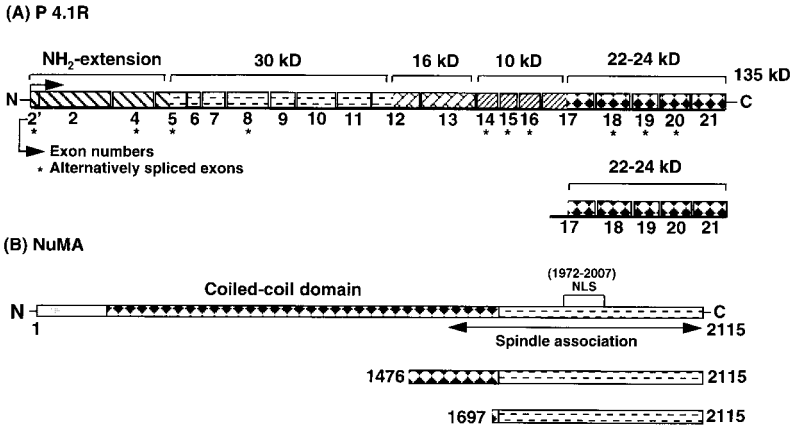

Figure 1. Schematic representation of 4.1R and NuMA peptides found to interact in the yeast two-hybrid screening of a human brain cDNA library. (A) The organization of protein 4.1R and its 22–24-kD domain peptides employed as bait in the yeast two-hybrid assays. Different exons of 4.1R gene that correspond to its different domains are shown. The asterisks represent the alternatively spliced exons. (B) A schematic representation of the structural organization of the NuMA protein and its segments encompassed by positive clones obtained as prey in the two-hybrid assays.

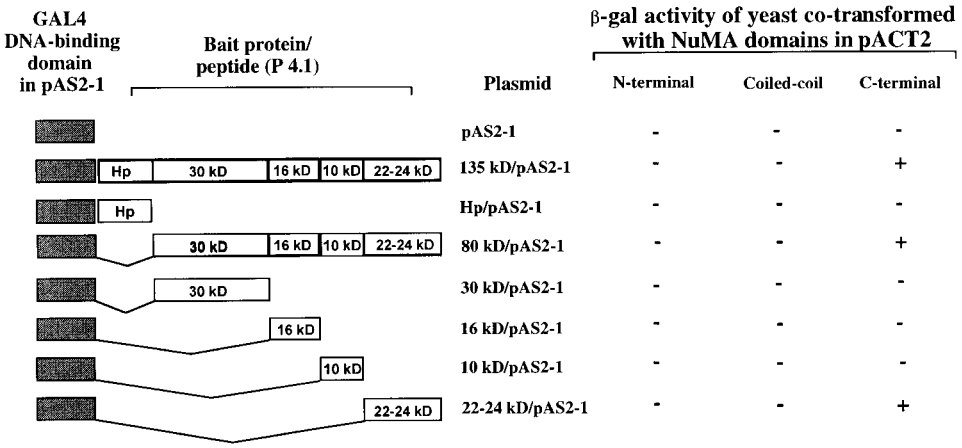

Figure 2. 4.1R and NuMA interact through their COOH-terminal domains. 4.1R and NuMA peptides and their segments were expressed as Gal4-BD or Gal4-AD fusion proteins, respectively, in yeast strain Y190 by cotransformation, and were assayed for the expression of the reporter genes (see Materials and Methods). (+) indicates the expression of the reporter genes, *LacZ* and *HIS3* (and thus the interaction between the peptides) and (−) indicates nonexpression of the reporter genes (and thus no interaction between the peptides).

4.1R as fusion products of Gal4-BD in pAS2-1 and cotransformed into Y190 with different domains of NuMA expressed as Gal4-AD fusion products in pACTII or pACT2, as shown in Fig. 2. The full-length (135-kD) 4.1R, its 80-kD isoform, and the 22–24-kD domain interacted with the COOH-terminal domain, but not the NH_2-terminal or the coiled-coil domain of NuMA, in the yeast two-hybrid assays. The HP, 30-, 16-, or 10-kD domain of 4.1R did not interact with NuMA. None of the domains of 4.1R, when expressed as Gal4-BD fusion proteins in Y190, expressed the reporter genes by themselves or in combination with the Gal4-AD in pACT2 alone (data not shown). These data suggest that the COOH-terminal domains of 4.1R and NuMA are necessary and sufficient for their interaction.

Interaction between 4.1R and NuMA Occurs through the COOH-terminal 62 Amino Acids of 4.1R and Amino Acids 1788–1810 of NuMA

To delineate the minimal sequences of 4.1R and NuMA that interact, we examined the interactions between different segments of the COOH-terminal domains of both the 4.1R and NuMA in the yeast two-hybrid assays, by expressing segments of the COOH-terminal domain of 4.1R as Gal4-BD fusion proteins and that of NuMA as Gal4-AD fusion proteins, respectively. Neither the deletion of the amino acids encoded by the part of exon 17 that belongs to the 22–24-kD domain of 4.1R (exons 18–21/pGBT9), nor the deletion of the amino acid residues encoded by exons 17, 18, and 19 (exons 20 and 21/pGBT9) affected the interaction between 4.1R and NuMA. Deletion of the peptide segment encoded by exon 21 (exons 18–20/pGBT9) abolished the interaction of 4.1R with NuMA, although this segment alone (exon 21/pGBT9) was not sufficient for binding to NuMA (Fig. 3 A). The amino acids encoded by exons 20 and 21 of 4.1R (exons 20 and 21/pGBT9) were sufficient for its association with NuMA. However, the amino acids encoded by exon 20 alone (exon 20/pGBT9) did not bind to NuMA. Therefore, the extreme COOH-terminal 62 amino acids of 4.1R derived from exons 20 and 21 are required and sufficient for its interaction with NuMA.

The two different clones of NuMA obtained from the yeast two-hybrid screening started at positions corresponding to amino acids 1476 and 1697 of NuMA. Peptide segments of NuMA representing amino acids 1476–1695 or 1697–1787 did not interact with 4.1R (Fig. 3 B). Deletion of NuMA sequences representing the amino acids from the COOH-terminal end to 1804 resulted in a weak interaction with 4.1R, whereas amino acids 1788–1810 showed substantial binding. Therefore, we believe that amino acids 1788–1810 of NuMA are necessary and sufficient for its interaction with 4.1R. These results suggest that the interaction between 4.1R and NuMA occurs through the amino acids encoded by exons 20 and 21 of 4.1R, and the amino acids 1788–1810 of NuMA.

Protein 4.1R Interacts with NuMA In Vitro

To confirm the direct interaction between 4.1R and NuMA, the association was further analyzed by an in vitro binding assay. GST-tagged 135-, 80-kD, and different domains (HP, 30, 16, 10, and 22–24 kD) of 4.1R were expressed and purified (Fig. 4 A). Because of the insolubility of the 30-kD peptide, there was minor host protein contamination in preparations of 30 kD/GST and 80 kD/GST fusion proteins (lanes 2 and 8, respectively). In 16 kD/GST preparation, another protein band, probably a degradation product, was also observed (lane 3). The authenticity of these fusion proteins was verified with the corresponding antibodies (data not shown). Immobilized GST-tagged 4.1R proteins were incubated with in vitro translated and [^{35}S]methionine-labeled NuMA peptides corresponding to amino acids 1697–2102 (NuMA1/TOPO), 1697–1889 (NuMA2/TOPO), or 1831–2102 (NuMA3/TOPO). Retention of NuMA peptides on the beads was analyzed by SDS-PAGE as described in Materials and Methods. The yeast two-hybrid assays showed that NuMA sequences required for its interaction with 4.1R are located within amino acid sequences 1788–1810. As shown in Fig. 4, B and C, both NuMA1/TOPO and NuMA2/TOPO that contained these sequences bound strongly to both 135-kD and 80-kD 4.1R isoforms and its 22–24-kD domain. The HP, 30-, 16-, and 10-kD domains, or GST alone did not bind to NuMA. NuMA3/TOPO, which did not contain these se-

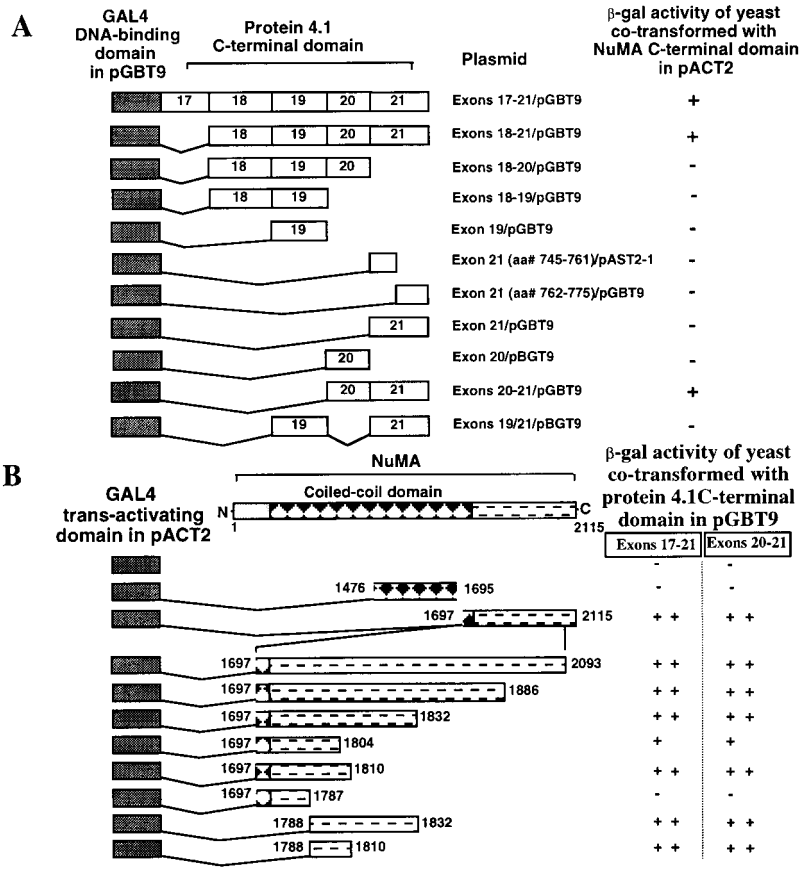

Figure 3. Interaction between 4.1R and NuMA occurs through the COOH-terminal 62 amino acids of 4.1R and amino acids 1788–1810 of NuMA. (A) Schematic diagrams of the various exons within the 22–24-kD domain of 4.1 (white rectangles) fused to the DNA binding domain of GAL4 (shaded rectangles) used in the two-hybrid assay are shown. The names of the plasmids that encoded each construct are given to the right of each schematic diagram. Plasmid pAS2-1 expresses the GAL4-BD alone and was used as a negative control. These plasmids were cotransformed with pACT2-NuMA expressing amino acids 1697–2115 of NuMA fused to the activation domain of Gal4. The column on the right shows the results of filter assays for β-galactosidase (β-gal) activity as the reporter gene: (+) indicates the expression of the reporter genes (and thus the interaction between the peptides) and (−) indicates nonexpression of the reporter genes (and thus no interaction between the peptides). The last 62 amino acids of protein 4.1 encoded by exons 20 and 21 bind to NuMA. (B) Schematic diagrams of the various NuMA polypeptides (dotted rectangles) fused to the activation domain of GAL4 (shaded rectangles) used in the two-hybrid assay are shown. Plasmid pACT2, which expresses the Gal4-AD alone, was used as a negative control. These plasmids were cotransformed with pGBT9 or pAS2-1 expressing COOH-terminal domain of 4.1R or its exons 20 and 21 fused to the DNA-binding domain of GAL4. The column on the right shows the results of filter assays for β-galactosidase activity: (++) indicates strong β-gal activity, (+) indicates detectable β-gal activity, and (−) indicates no detectable β-gal activity.

quences, did not bind to any of the 4.1R/GST fusion proteins (Fig. 4 D). These results are consistent with that of the yeast two-hybrid assays, and further confirm the specific interaction between 4.1R and NuMA.

Expression and Subcellular Localization of 4.1R and NuMA in HeLa and MDCK Cells

We verified the expression of 4.1R and NuMA in HeLa and MDCK cells, both at the RNA and protein level. A 6.5-kb 4.1R mRNA and a 7.2-kb NuMA mRNA were detected in HeLa and MDCK cells (data not shown). Affinity-purified antibodies specific for HP (anti-HP 4.1) recognized four different protein bands in cytoplasmic protein fraction (molecular masses of ~35, 45, 76, and 135 kD), two bands in the nuclear protein fraction (35 and 135 kD), and a major 135-kD band in the nuclear matrix fraction of MDCK cells (Fig. 5 B). However, no protein band was seen when a replica blot was immunoblotted with the preimmune serum (Fig. 5 A).

To verify that the protein bands recognized by anti-HP 4.1 Ab are also recognized by other anti-4.1R antibodies, replica blots were also immunoblotted with antibodies specific for exon 19 (Fig. 5 C, anti-24 kD Ab) and the 10-kD domain of 4.1R (Fig. 5 D, anti-10 kD Ab). In addition to other bands, both the anti-24 kD Ab and anti-10 kD Ab recognized a predominant 135-kD protein band in cytoplasmic, nuclear, and nuclear matrix fractions, suggesting that the 135-kD protein band recognized by the anti-HP 4.1 Ab is 4.1R. Unexpectedly, the anti-24 kD Ab did not show an 80-kD band in any of the fractions. This could have resulted from a low level of expression of 80 kD isoform(s) containing exon 19 in these cells. The anti-10 kD Ab revealed the presence of both the 135- and 80-kD 4.1R isoforms in the cytoplasmic and nuclear fractions, and additionally detected the presence of 4.1R epitopes in a predominant ~110 kD polypeptide that was localized both in the cytoplasmic and nuclear fractions (Fig. 5 D). Similar distribution of 4.1R was observed in MDCK cells using anti-HP 4.1 antibodies and immunofluorescent staining (data not shown). The 135-kD isoform of 4.1R was predominantly localized in the nucleus, centrosomes, and to some extent at the points of cell–cell contact of interphase cells. Also, it was localized in the mitotic spindle and spindle poles of dividing cells. These results are consistent with recent studies that 4.1R epitopes are localized in centrosomes, nucleus, and nuclear matrix (Krauss et al., 1997a,b).

The anti-NuMA antibodies recognized two immunoreactive bands of ~210 and 230 kD in the nuclear and nu-

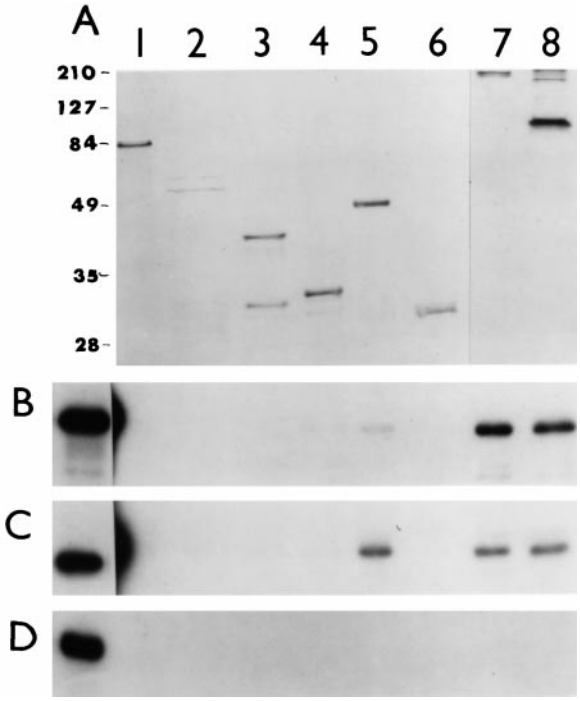

Figure 4. Protein 4.1R interacts with NuMA in vitro. Purified bacterially produced 4.1R/GST fusion proteins were incubated with [^{35}S]methionine-labeled NuMA. After incubation, the bound protein complexes were analyzed by SDS-PAGE and visualized by fluorography. (A) Purified 4.1R/GST fusion proteins are shown by Coomassie blue staining: HP/GST (lane 1); 30 kD/GST (lane 2); 16 kD/GST (lane 3); 10 kD/GST (lane 4); 24 kD/GST (lane 5); GST (lane 6); 135 kD/GST (lane 7); and 80 kD/GST (lane 8). (B) Binding of 4.1R/GST to [^{35}S]methionine-labeled NuMA amino acid sequence 1697–2102 translated from NuMA1/TOPO is shown. (C) Binding of 4.1R/GST to NuMA amino acid sequence 1697–1889 translated from NuMA2/TOPO. (D) Binding of 4.1R/GST to NuMA amino acid sequence 1831–2102 translated from NuMA3/TOPO. The input [^{35}S]methionine-labeled NuMA proteins are shown on the far left lane.

clear matrix protein fractions, and a 210-kD band in the cytoplasmic fraction (Fig. 5 E). This is consistent with the observation that two different isoforms of NuMA are expressed in interphase cells (Zeng et al., 1994). Previously published results (Yang and Snyder, 1992) suggest that NuMA is localized in the nucleus of interphase cells. We believe that the minor fraction of NuMA seen in the cytoplasmic fraction (Fig. 5 E) occurred because of some cell nuclei lysis during cell fractionation.

To assess the quality of subcellular fractionation and document that the 4.1R isoform(s) seen in the nuclear and nuclear matrix fractions are not due to contamination of cytoplasmic proteins, different subcellular fractions were also immunoblotted with antibodies to proteins known to localize in cell cytoplasm (Pfarr et al., 1990; Zahler et al., 1993). Immunoblotting with anti–Na^+-K^+-ATPase α1 Ab (Fig. 5 F) and antidynein mAb (Fig. 5 G) detected an ~120- and 70-kD band, respectively, only in the cytoplasmic fraction. This suggests that the 4.1R isoform(s) seen in the nuclear and nuclear matrix fractions is not the result of contamination of 4.1R isoforms localized in the cytoplasm.

Because 4.1R isoforms are documented to associate with centrosomes at mitosis and even after microtubule depolymerization (Krauss et al., 1997a; Krauss, S.W., R. Heald, G. Lee, T. Berger, and J.A. Chasis. 1998. *Mol. Biol. Cell.* [Suppl.] 9:294a), different subcellular fractions were immunoblotted with antibodies for centrosomal markers such as γ-tubulin (Stearns and Kirschner, 1994) and pericentrin (Doxsey et al., 1994). This was done to abrogate the possibility that 4.1R isoforms seen in nuclear and nuclear matrix fractions could have occurred because of centrosomal contamination in these fractions. Immunoblotting of different subcellular fractions with anti–γ-tubulin Ab (Fig. 5 H) and antipericentrin Ab (Fig. 5 I) showed the corresponding 48-kD γ-tubulin and 220-kD pericentrin only in cytoplasmic fraction. This suggests undetectable centrosomal contamination in the nuclear and nuclear matrix fractions.

4.1R and NuMA Colocalize at Different Stages of the Cell Cycle

To further localize the association of 4.1R and NuMA, we used double immunofluorescence staining and confocal microscopy, and analyzed MDCK cells at different stages of cell cycle (see Materials and Methods). In interphase, 4.1R epitopes were found diffusely in the cytoplasm, but predominantly inside the nucleus (Fig. 6 A1, green), whereas NuMA epitopes were found mainly inside the nuclei (Fig. 6 A2, red). In mitotic cells, both 4.1R and NuMA intensely stained the spindle poles as shown in B1/C1 and B2/C2, respectively. NuMA epitopes were mainly found in the nucleus of the newly forming daughter cells (D2), whereas 4.1R epitopes were seen in the cytoplasm as well as nuclei (D1). The yellow color produced by superimposing green and red suggested that some 4.1R and NuMA epitopes colocalized in interphase nuclei (A3), in the mitotic spindle and spindle poles (B3 and C3), and in the daughter cell nuclei (D3). Krauss et al. (1997b), using immunogold staining and electron microscopy, observed that in the interphase 4.1R epitopes were primarily found in the vicinity of dense bodies. Zeng et al. (1994) reported that NuMA was present in subsets of the core filaments. However, using double label immunofluorescent staining and confocal microscopy, Krauss et al. (1997b) also observed 4.1R foci within the nuclear area that was stained by NuMA. These results, the diffuse distribution of 4.1R during interphase and its segregation at the spindle poles during mitosis, are similar to those of other proteins essential for mitotic spindle assembly such as, NuMA (Price and Pettijohn, 1986), dynein (Steur et al., 1990), HSET (Kuriyama et al., 1995), and Eg5 (Houliston et al., 1994).

Protein 4.1R and NuMA Associate In Vivo

To determine whether the native 4.1R interacts with NuMA in vivo, coimmunoprecipitation assays were performed using nuclear extracts from MDCK cells. MDCK nuclear extracts were subjected to coimmunoprecipitation with anti-HP 4.1 or anti-NuMA antibodies. Coprecipitated polypeptides were detected by immunoblot staining using anti-HP 4.1 or anti-NuMA antibodies. As shown in Fig. 7, A and B, when preimmune serum, mouse IgG, or anti-p53 mAb were used to immunoprecipitate MDCK nuclear ex-

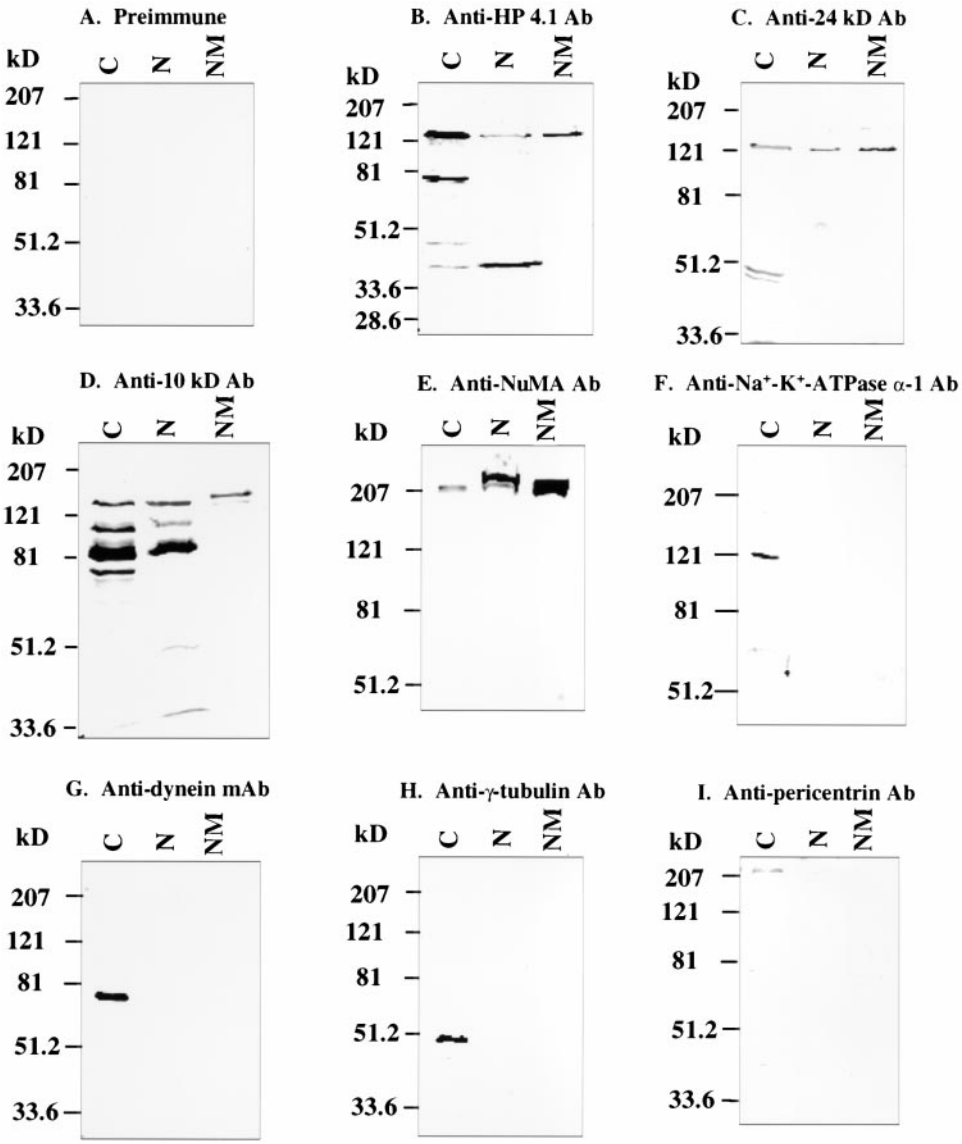

Figure 5. Subcellular localization of 4.1R and NuMA proteins. MDCK cells were fractionated and 20 μg proteins (unless mentioned otherwise) from the cytoplasm (equivalent to ~1.6×10^5 cells), nuclear (equivalent to ~5×10^5 cells), and nuclear matrix (equivalent to ~7×10^6 cells) fractions were analyzed as described in Materials and Methods. Shown are immunoblots of preimmune serum for anti-HP 4.1 Ab (A), anti-HP 4.1 Ab (B), anti-24 kD Ab (C), anti-10 kD Ab (D), anti-NuMA Ab (E), anti–Na^+-K^+-ATPase α1 Ab (F), antidynein mAb (G), and anti–γ-tubulin Ab (H). The blot in I was immunoblotted with antipericentrin Ab and was loaded with 50 μg proteins from each fraction.

tracts, neither 4.1R (Fig. 7 A, lanes 2, 4, and 5) nor NuMA (Fig. 7 B, lanes 2, 4, and 5) precipitated. However immunoprecipitation with anti-HP 4.1 Ab and subsequent detection with anti-NuMA mAb showed that anti-HP 4.1 Ab precipitates two proteins of ~210 and 230 kD (Fig. 7 B, lane 1). These proteins comigrate with two polypeptides of similar molecular mass that were immunoprecipitated by anti-NuMA mAb (Fig. 7 B, lane 3) and were detected by immunoblotting using anti-NuMA antibodies. Similarly, immunoprecipitation with anti-NuMA mAb precipitated a protein of ~135 kD (Fig. 7 A, lane 3) that comigrates with 4.1R and immunoreacts with anti-HP 4.1 Ab (Fig. 7 A, lane 1).

These results suggest that a nuclear isoform of 4.1R and NuMA is contained in the same protein complex in vivo. The coprecipitation of NuMA by anti-HP 4.1 antibodies, detection of 4.1R that coprecipitated with NuMA by anti-HP 4.1 antibodies, and the molecular mass of 4.1R isoform that coprecipitates with NuMA (135 kD) suggest that the 4.1R isoform(s) that interact(s) with NuMA is a 135-kD isoform that contains HP observed in nonerythroid isoforms. To find out which fractions of 4.1R and NuMA associate together, the efficiencies of immunoprecipitation and coprecipitation were determined. Quantitation from Fig. 7, A and B, shows that ~29% of 4.1R was precipitated by anti-HP 4.1 Ab (that coprecipitated ~8% NuMA) and ~47% of NuMA was precipitated by anti-NuMA mAb which in turn brought down ~15% 4.1R. The limited efficiency of the immunoprecipitation could have happened because of insufficient amounts of antibodies in the immunoprecipitation assays. The lower efficiency of coimmunoprecipitation as compared with that of the immunoprecipitation suggests that only a fraction of these molecules associate together in vivo. This is consistent with the subcellular fractionation and immunofluorescent staining results.

To examine the possible interaction of other isoforms of 4.1R with NuMA, immunoprecipitates of anti-NuMA mAb were also analyzed by antibodies specific to other domains of 4.1R. Analysis of anti-NuMA mAb immuno-

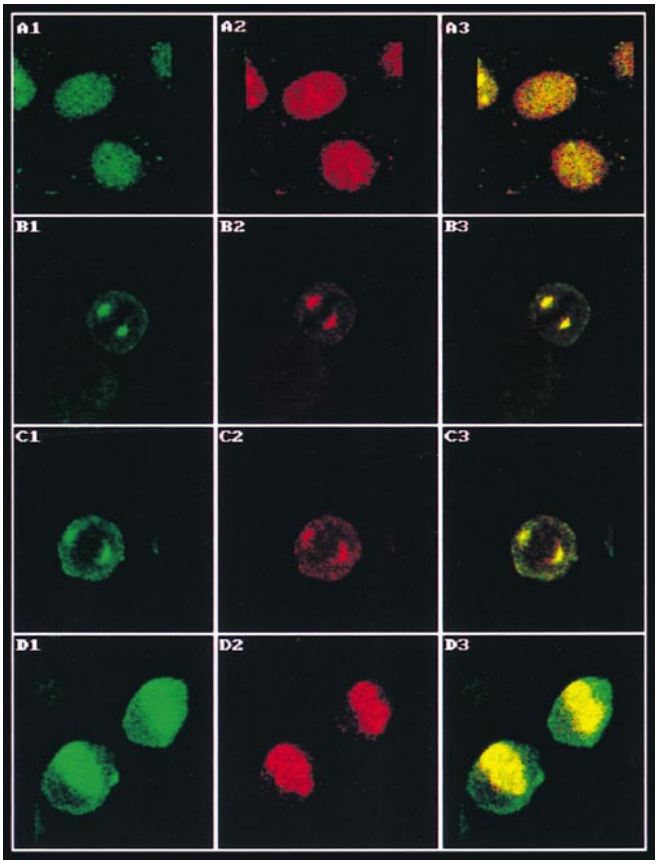

Figure 6. Subcellular colocalization of protein 4.1 and NuMA at various cell cycle stages of MDCK cells. MDCK cells were fixed and processed for immunofluorescence with protein 4.1R and NuMA antibodies as described in Materials and Methods. Shown are cells in interphase (A1–A3), mitotic (B1–B3 and C1–C3), and late mitotic stages (D1–D3). Green represents anti-HP 4.1 epitopes, red represents anti-NuMA epitopes, and yellow indicates the colocalization of protein 4.1R and NuMA.

precipitates by immunoblotting using anti-24 kD Ab (data not shown because of the poor quality of the immunoblot) revealed the presence of the 135-kD 4.1R isoform that was detected by using anti-HP 4.1 Ab, but did not show the presence of other isoforms of 4.1R. These data further support our contention that a 135-kD isoform(s) of 4.1R interacts with NuMA, although it does not eliminate the possibility that other isoforms of 4.1R or 4.1-like genes also interact with NuMA. Because actin, spectrin, and calmodulin were reported to interact with 4.1R, immunoprecipitates of anti-HP 4.1 Ab and anti-NuMA mAb were also analyzed by Western blot using antibodies specific to these proteins. However, actin (Fig. 7 C), spectrin (Fig. 7 D), or calmodulin (data not shown) was not detected in the immunoprecipitates of anti-HP 4.1 Ab and anti-NuMA mAb.

4.1R Associates with Mitotic Spindle Pole Organizing Proteins such as NuMA, Dynein, and Dynactin

To test whether 4.1R might also reside in association with other components of the mitotic apparatus, we asked whether it could be found in association with other proteins that are known to organize the mitotic spindle and spindle poles. Increasing evidence from different laboratories suggests that NuMA, dynein, and dynactin form a complex that organizes the spindle pole and stabilizes the mitotic spindle (Merdes et al., 1996; Gaglio et al., 1997; Heald et al., 1997). Therefore, we enriched cells with mitotic spindle and spindle pole organizing components by using double thymidine block and nocodazole. Afterwards, we performed immunoprecipitation in highly synchronized mitotic HeLa extracts using anti-HP 4.1 Ab, anti-NuMA mAb, antidynein (70.1) mAb, or antidynactin ($p150^{glued}$, the 150-kD component of the dynactin complex) mAb. We looked for the presence of 4.1R, NuMA, dynein, and dynactin in the immunoprecipitates by immunoblot. An irrelevant antibody (anti-p53 mAb) and mouse IgG were used as controls for mAbs. To rule out nonspecific aggregation as a basis for detecting proteins on immunoblots and in the immunoprecipitates, preimmune rabbit serum was used as a control for Abs.

As shown in Fig. 8 A, ~140-kD protein, immunoprecipitated and detected by immunoblot staining using anti-HP 4.1 Ab (lane 1), coprecipitated with NuMA, dynein, and dynactin (lanes 3, 5, and 6, respectively) when immunoprecipitation was performed with anti-NuMA mAb, antidynein mAb or antidynactin mAb ($p150^{glued}$), but not by preimmune serum, anti-p53 mAb, or mouse IgG (lanes 2, 4, and 7, respectively). This ~140 kD protein corresponds to a 135-kD 4.1R isoform. We previously have documented such an alteration in the mobility of 135-kD isoforms of 4.1R because of phosphorylation at mitosis (Huang, S.C., S.N. Mattagajasingh, and E.J. Benz, Jr. 1997. *Blood*. 90: 269a). When these blots were stripped and reprobed with antibodies specific for the COOH-terminal 24-kD domain of 4.1R (Fig. 8 B, anti-24 kD Ab), the ~140-kD protein was seen in the immunoprecipitates of anti-HP 4.1, anti-NuMA mAb, antidynein mAb, and antidynactin mAb (lanes 1, 3, 5, and 6, respectively). No other convincing protein band corresponding to other 4.1R isoforms was seen in the immunoprecipitates (data not shown). Thus, other isoforms of 4.1R are unlikely to associate with NuMA, dynein, or dynactin.

When a replica of the blot in Fig. 8 A was probed with anti-NuMA mAb (Fig. 8 C), an ~230-kD band corresponding to NuMA was seen in the immunoprecipitates of anti-HP 4.1 Ab, anti-NuMA mAb, antidynein mAb, and antidynactin ($p150^{glued}$) mAb (lanes 1, 3, 5, and 6, respectively), but not in any of the control antibodies (lanes 2, 4, and 7). Similar analysis with appropriate antibodies detected dynein (Fig. 8 D) and dynactin (Fig. 8 E) in immunoprecipitates of anti-HP 4.1 Ab, anti-NuMA mAb, antidynein mAb, and antidynactin ($p150^{glued}$) mAb (lanes 1, 3, 5, and 6, respectively), but not in any of the control antibodies used for immunoprecipitation (lanes 2, 4, and 7). These results suggest that 4.1R, NuMA, dynein, and dynactin associate together in vivo, and nonspecific aggregation is unlikely to account for this association.

We attempted to quantitate how much 4.1R associates with NuMA and vice versa, by analyzing the efficiencies of their immunoprecipitation and coprecipitation. Representative immunoblots of anti-HP 4.1 Ab and anti-NuMA mAb immunoprecipitated, along with the supernatant fractions probed with anti-HP 4.1 Ab and anti-NuMA

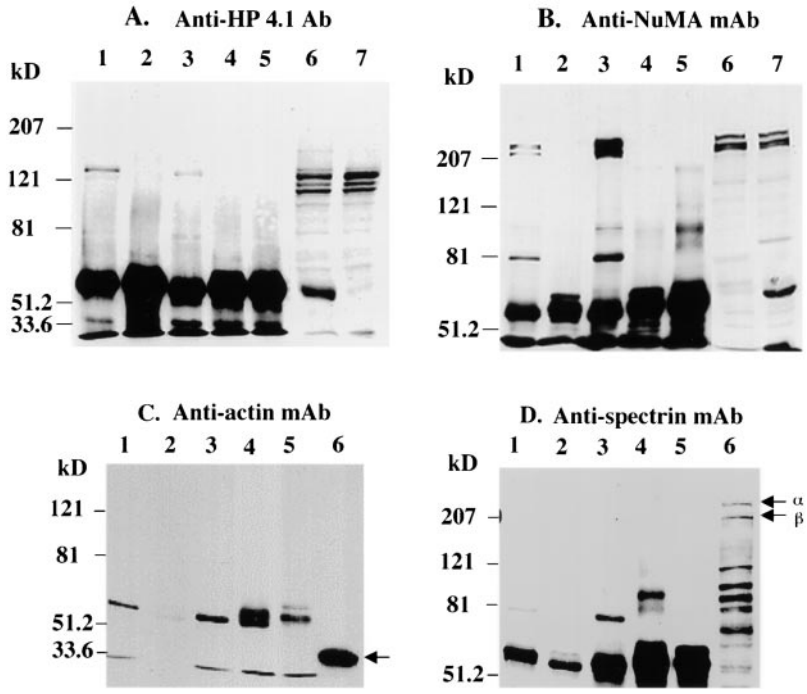

Figure 7. Coimmunoprecipitation of 4.1R and NuMA from MDCK cell nuclear extracts. MDCK nuclear extracts were prepared as described in Materials and Methods and were subjected to immunoprecipitation using different antibodies. The immunoprecipitates were analyzed by immunoblotting using anti-HP 4.1 Ab (A), anti-NuMA mAb (B), antiactin mAb (C), or antispectrin mAb (D). In A and B, lanes 1–5 were loaded with one-fourth of the immunoprecipitates of anti-HP 4.1 Ab (lane 1), preimmune serum (lane 2), anti-NuMA mAb (lane 3), anti-p53 mAb (lane 4), and mouse IgG (lane 5). Lanes 6 and 7 were loaded with one-ninth of the supernatant fractions of anti-HP 4.1 Ab and anti-NuMA mAb immunoprecipitates, respectively. For C and D, lanes 1–5 were the same as in A, and lane 6 was loaded with 40 μg of cell lysate. Arrows indicate the position of actin in C and that of α- and β-spectrin in D.

mAb, are shown in Fig. 8, F and G, respectively. Quantitation of the efficiencies of immunoprecipitation and coprecipitation from these blots showed that ~39% of total cellular 135-kD 4.1R protein was immunoprecipitated by anti-HP 4.1 Ab which in turn brought down 11% of total cellular NuMA. Similarly, ~31% of NuMA protein was immunoprecipitated by anti-NuMA mAb that brought down ~10% of 4.1R. It appears that under these conditions about one-third of NuMA and 4.1R remain associated and antibodies, not the antigens, are the limiting factors.

To document the presence of other candidate proteins in this protein complex, anti-HP 4.1 Ab immunoprecipitates were examined by gel electrophoresis and silver staining (Fig. 8 H). Approximately 15 prominent protein bands of very high molecular mass (~40 kD) could be seen on the gel. Protein bands that correspond to NuMA, the $p150^{glued}$ subunit of the dynactin complex, 135-kD 4.1R, and dynein in immunoblots were seen on the gel and are indicated in Fig. 8 H. To obtain an estimate of the molar ratios between NuMA, 4.1R, dynein, and dynactin in the immunoprecipitated protein complex, we determined the relative amounts of these proteins by scanning a picture of the silver stained gel of anti-HP 4.1 Ab immunoprecipitates. The molar ratio between NuMA, 4.1R, and dynactin was found to be 1:1.3:1.4. The molar ratio of the dynein intermediate chain could not be determined because of higher background staining. However, different proteins may not stain equally by silver stain (Sammons et al., 1981). To examine the possible participation of other proteins in this protein complex, known to bind to 4.1R such as actin, spectrin, and calmodulin, we analyzed the anti-HP 4.1 Ab and anti-NuMA mAb immunoprecipitates by immunoblot staining using antibodies specific for these proteins (data not shown). Neither actin, spectrin, nor calmodulin was detected in these immunoprecipitates. The identities of other proteins remain to be identified.

Our results extend the previous findings by Merdes et al. (1996) and Gaglio et al. (1997). Merdes et al. (1996) presented persuasive evidence of dynein and dynactin in a mitotic spindle complex with NuMA. Gaglio et al. (1997) also showed that microinjection of dynein-specific antibody blocked spindle formation by inhibiting the association of dynein with the mitotic spindle and dislocating NuMA from spindle poles. Our findings are consistent with these observations and suggest that 4.1R, at least a 135-kD isoform, also participates in this complex. Results obtained with control antibodies, coupled with the nondetection of actin, spectrin, and calmodulin, support the contention that our coimmunoprecipitation results are far more likely to reflect a specific association in vivo rather than a nonspecific aggregation artifact of the immunoprecipitation assays.

Overexpression of 135^{++}kD/GFP Alters the Localization of NuMA

Because the above results suggested that there may be a direct interaction between NuMA and 4.1R, and NuMA is a nuclear protein, and is known to play a role in the organization of mitotic spindle and spindle poles, we asked if overexpression of 4.1R could disturb the intracellular distribution of NuMA or disrupt the organization of mitotic spindle and spindle poles. We transiently transfected HeLa cells with 135^{++}kD/GFP construct and examined the effect of overexpression of this isoform on transfected cells. Different degrees of 135^{++}kD/GFP expression were observed in transfected cells 10 h after transfection. In most of the transfected cells, 135^{++}kD/GFP fusion proteins were located in the cytoplasm. However, in some

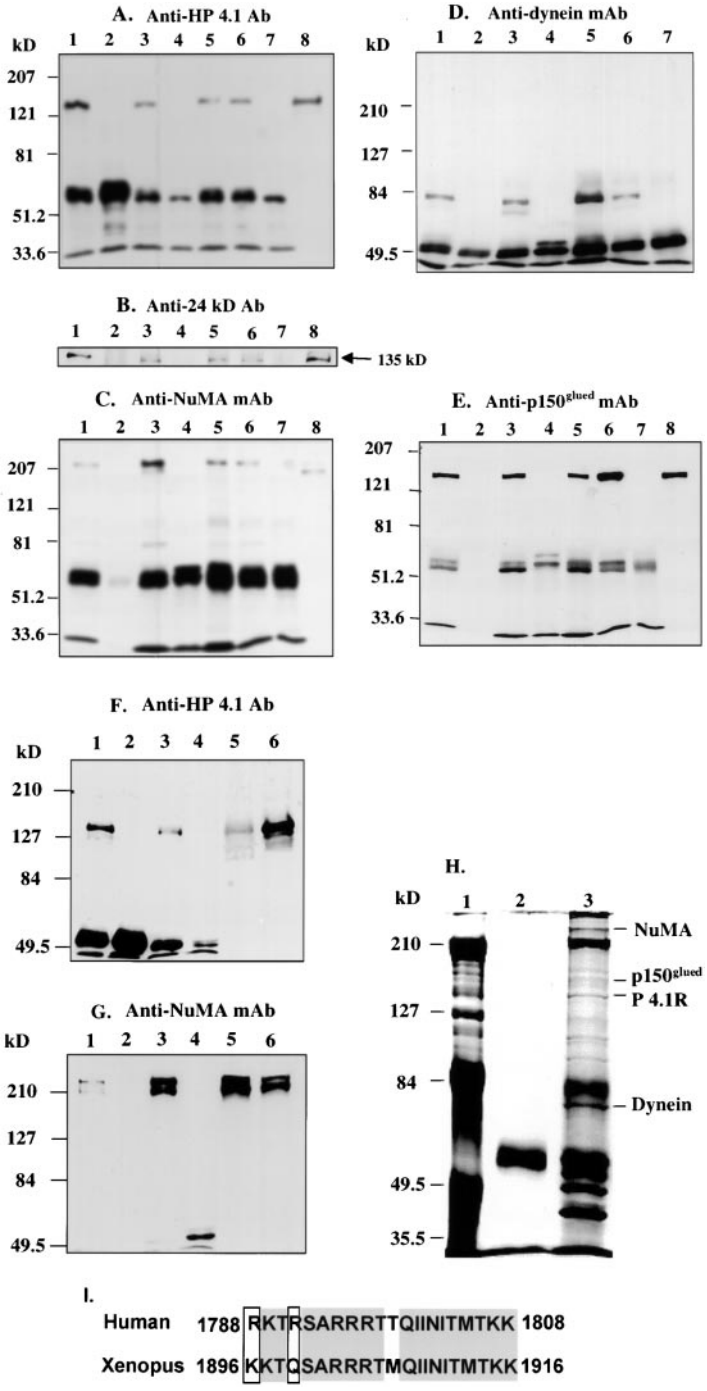

Figure 8. Immunoprecipitaion from mitotic HeLa extracts reveals that 4.1R associates with NuMA, cytoplasmic dynein, and dynactin. Mitotic HeLa nuclear extracts were prepared as described in Materials and Methods, and were subjected to immunoprecipitation using different antibodies. The immunoprecipitates were analyzed by immunoblotting using anti-HP 4.1 Ab (A and F), anti-24 kD Ab (B), anti-NuMA mAb C and G, antidynein mAb (D), or anti-p150^{glued} mAb (E). In A–C, lanes 1–7 were loaded with one fourth of the immunoprecipitates of anti-HP 4.1 Ab (lane 1), preimmune serum (lane 2), anti-NuMA mAb (lane 3), anti-p53 mAb (lane 4), antidynein mAb (lane 5), antidynactin mAb (lane 6), and mouse IgG (lane 7), respectively. Lane 8 was loaded with 40 μg of mitotic HeLa lysate. The anti-24 kD Ab recognized the 135-kD 4.1R isoform in the immunoprecipitates and is indicated by an arrow. The blot in D was probed with antidynein mAb and lanes 1–7 were the same as A. The blot shown in E is the same as C except that it was stripped and reprobed with antidynactin mAb. Lanes 1–4 of the blot in F were loaded with one-fourth of the immunoprecipitates of anti-HP 4.1 Ab (lane 1), preimmune serum (lane 2), anti-NuMA mAb (lane 3), and mouse IgG (lane 4), respectively. Lanes 5 and 6 were loaded with one-ninth of the supernatant fractions of anti-HP 4.1 Ab and anti-NuMA mAb immunoprecipitates, respectively. The blot shown in (G) is the same as in (F) except that it was stripped and reprobed with anti-NuMA mAb. H is a silver-stained gel of molecular mass markers (lane 1), immunoprecipitates of preimmune serum (lane 2), and anti-HP 4.1 Ab (lane 3). The migration position of 4.1R (135-kD isoform), NuMA, dynein, and dynactin are indicated in the figure. Shown in I is the alignment of NuMA residues essential for its binding to 4.1R in human and *Xenopus* (available in GenBank/EMBL/DDBJ under accession number Y07624). The identical residues shared by the two proteins are shaded and the conserved amino acid changes are boxed. Numbers represent the position of respective amino acids. The peptide segment of NuMA that interacts with 4.1R is highly conserved across the species.

cells the fusion proteins could be detected in both cytoplasm and nucleus.

As shown in Fig. 9, GFP proteins were expressed in both nuclei and cytoplasm of pEGFP-C1 vector transfected HeLa cells (A1), whereas NuMA was exclusively localized in the nuclei (A2). This suggests that the expression of GFP protein alone did not alter the localization of NuMA in the interphase nuclei. In contrast, nearly all of the cells that strongly expressed 135^{++}kD/GFP, regardless of whether the localization of 135^{++}kD/GFP was in the nucleus or cytoplasm, showed altered localization of NuMA in the interphase cells. NuMA was distributed diffusely in these cells (Fig. 9, B2 and C2). The dispersed localization of NuMA was not apparent in the cells that weakly expressed the fusion protein, as shown in the top cell of Fig. 9 B2. In the cells that expressed the GFP alone, the localization of NuMA appeared to be defined by a boundary. In cells that highly expressed the 135^{++}kD/GFP fusion protein, NuMA was diffusely distributed well beyond this boundary. As shown in Fig. 9, B3 and C3, there was an enhancement of yellow in the cytoplasm due to a combination of green and red, indicating the colocalization of these two proteins. This possibly suggests the appearance of 4.1R and NuMA complex aggregates in the cytoplasm,

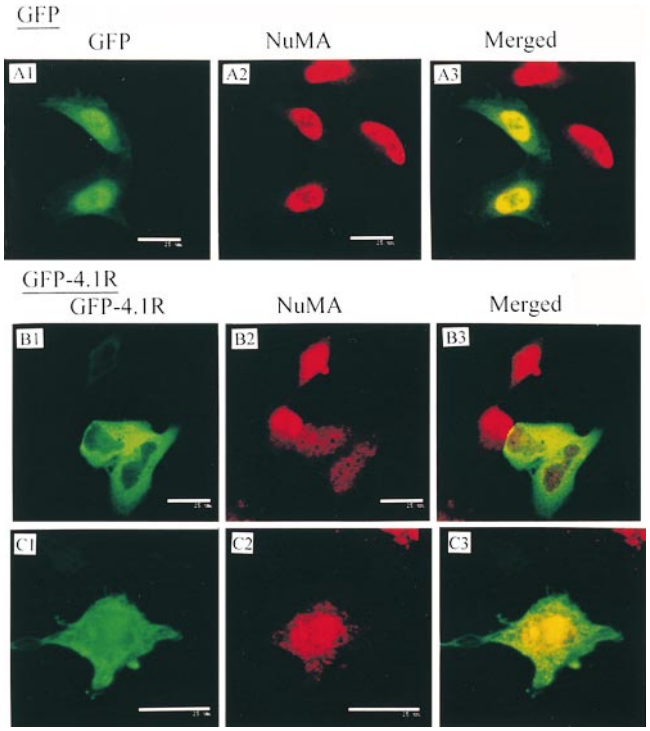

Figure 9. Alteration in the localization of NuMA in 135^{++}kD/GFP 4.1R-transfected HeLa cells. HeLa cells transfected with pEGFP or 135^{++}kD/GFP were fixed and processed for immunofluorescence with NuMA antibody and visualized for the localization of GFP (green) and NuMA (red). Shown are cells transfected with pEGFP (A1–A3) or 135^{++}kD/GFP (B1–B3 and C1–C3). Green represents fluorescence of GFP, red represents anti-NuMA epitopes, and yellow indicates the colocalization of GFP and NuMA.

where it is not found normally. Conversely, there was a loss of staining signal in the nucleus in Fig. 9 B2 as compared with nuclear staining of NuMA in vector/nontransfected cells.

These results suggest that overexpression of 135^{++}kD 4.1R isoform in HeLa cells may disrupt the organization of nuclear structural proteins such as NuMA. Cells that strongly expressed 135^{++}kD/GFP also gradually disappeared from the population 3–4 d after transfection. Furthermore, the selected disappearance of cells that expressed the fusion protein raised the possibility that such disruption of the normal organization of NuMA could adversely affect the cell cycle progression since the majority of 135^{++}kD/GFP overexpressed cells were eliminated from the cell population. It is interesting to note that we have not been able to identify cells that strongly overexpress the 135^{++}kD/GFP fusion protein in mitosis, whereas cells that expressed the fusion protein very weakly have been detected in mitosis. This preliminary observation is consistent with the notion that overabundance of 135^{++}kD 4.1R could indeed disrupt mitosis.

Discussion

Previous studies, from our laboratory and others (Tang et al., 1990; Conboy et al., 1991), have documented the existence of multiple isoforms of 4.1R. Many of these isoforms are present in nonerythroid cells; their function and biological significance remain unknown. In this study, we demonstrate an interaction between a 135-kD nonerythroid isoform of 4.1R and NuMA by using a yeast two-hybrid system, in vitro binding assays, coimmunoprecipitation, and immunocolocalization studies. We also show that a 135-kD 4.1R isoform resides in association with mitotic spindle pole organizing proteins such as NuMA, cytoplasmic dynein, and dynactin. Furthermore, overexpression of a 135-kD isoform alters the normal distribution of NuMA in interphase cells that gradually disappear from the cell population. Taken together, our results suggest that a 135-kD 4.1R isoform interacts with NuMA in the nucleus and nuclear matrix of interphase cells and in the mitotic spindle and spindle poles of dividing cells.

While the results in this paper strongly indicate that one or more isoforms of protein 4.1R interact with NuMA, they do not precisely pinpoint any one of the many potential forms that can be generated by alternative splicing of the 4.1R pre-mRNA. We believe that the isoforms of 4.1R that interact with NuMA are in the 135-kD molecular mass class even though NuMA interacts with both the 135-kD and 80-kD 4.1R isoforms in both yeast two-hybrid assays and in vitro binding assays. Indeed, NuMA does not even interact directly with HP that distinguishes the two molecular mass classes. These findings are compatible because the artificially created proximity between binding sites on each molecule in in vitro assays can allow them to interact, even if compartmentalization of the molecules, their proximity, orientation, posttranslational modification, or the presence of modifying factors precluded their interaction in intact cells. Further studies with additional domain specific antibodies and RT-PCR methods will be needed to define the exact isoform(s) that interact(s) in vivo.

Our studies are the first that specifically implicate protein 4.1R, as opposed to other members of the protein 4.1 family (4.1G, 4.1N, 4.1B), as a likely participant in mitosis. Previous studies from our group (Marchesi, V.T., S. Huang, T.K. Tang, and E.J. Benz, Jr. 1990. *Blood*. 76:12a) and from other laboratories have reported similar localization and redistribution of 4.1 in a cell cycle–dependent manner. Krauss et al. (1997a,b) reported the presence of 4.1 in centrosomes. Additionally, using double label electron microscopy, they have shown the colocalization of epitopes for 4.1 and the centrosome-specific autoimmune serum, 5051. Lallena and Correas (1997) also reported the localization of 4.1R in the nucleus. However, a recent study (Walensky et al., 1998) showed that epitopes for antibodies used in the above studies could be found among other protein 4.1 family members. In this present study, antibodies specific to HP, which is unique to 4.1R, were used. Therefore, our results strongly suggest that 4.1R isoform(s) containing HP localize(s) in the nucleus, centrioles, spindle fibers, and other intranuclear structures.

Differential localization of isoforms of other cytoskeletal proteins has been reported. For example, isoforms of band 3 with different 5′ ends localize either to peripheral membranes or to perinuclear regions (Cox et al., 1995). β-Spectrin isoforms with different COOH termini also exhibit different subcellular localization and binding part-

ners (Malchiodi-Albedi et al., 1993). Since the localization and redistribution of 4.1R during cell division that we have described here are similar to that of NuMA (Price and Pettijohn, 1986), these are consistent with its interaction with NuMA, and imply a novel location and role for a cytoskeletal protein.

NuMA is well known for its role in the formation and stabilization of the mitotic spindle (Kallajoki et al., 1991; Compton et al., 1992; Yang and Snyder, 1992; Gaglio et al., 1995). Accumulation of NuMA has been shown at the mitotic spindle poles and at or near the minus ends of microtubules (Kallajoki et al., 1991; Yang and Snyder, 1992; Maekawa and Kuriyama, 1993; Tang et al., 1993; Dionne et al., 1999). Sequestration of endogenous NuMA by microinjection of anti-NuMA antibodies into mitotic cultured cells was shown to disrupt the bipolar mitotic spindles (Kallajoki et al., 1991; Yang and Snyder, 1992; Gaglio et al., 1995). The spindle association activity of NuMA was mapped to its COOH-terminal part (residues 1538–2115; Tang et al., 1994). A recent study showed that the critical sequences for spindle pole localization are contained within the amino acid residues 1750–1800 of NuMA (Gueth-Hallonet et al., 1996). However, the mechanism by which NuMA binds to microtubules is not clear. NuMA does not contain a microtubule-binding region of kinesin (Yang et al., 1989), Map2 (Lewis et al., 1988), or tau (Himmler et al., 1989), but has been shown to directly bind to tubulin and organize microtubule formation through the distal portion of its COOH-terminal domain (Merdes et al., 1996).

Because 4.1R does not contain a microtubule-binding motif, it is possible that the localization of 4.1R at the mitotic spindle and spindle poles occurs through its interaction with NuMA or other proteins that directly bind to microtubules. Two other proteins, dynein and dynactin, with whom NuMA has been shown to form a complex at mitosis (Merdes et al., 1996), also possess microtubule binding domains (Waterman-Storer et al., 1995; Gee et al., 1997) and have been shown to organize and stabilize mitotic spindle and spindle poles (Gaglio et al., 1996, 1997). In a recent study, microinjection of dynein-specific antibodies into intact cells or immunodepletion of dynein from mitotic extracts disturbed assembly of mitotic spindle or mitotic aster, respectively, and prevented accumulation of NuMA at the spindle poles (Gaglio et al., 1997), suggesting that the accumulation of NuMA at the spindle poles may be dynein-dependent. However, the precise mechanism of organization of the microtubules or the localization of NuMA at the polar ends of the mitotic spindle is not clear.

Several lines of evidence support individual roles for NuMA, dynein, and dynactin. In an immunodepletion experiment using frog extracts, Merdes et al. (1996) observed a complex between NuMA, cytoplasmic dynein, and dynactin; the depleted extract failed to assemble normal mitotic spindles. Although this observation suggests that the complex between NuMA, cytoplasmic dynein, and dynactin may have a role in the assembly and stabilization of mitotic spindle, NuMA was found to associate with microtubules independent of cytoplasmic dynein (Gaglio et al., 1996). Additionally, organization of microtubules to astral arrays and association of NuMA and dynactin with the microtubules of the astral arrays have been observed in mitotic HeLa extracts from which cytoplasmic dynein (a minus end–directed motor protein) and Eg5 (a plus end–directed motor protein) were depleted. This suggests that NuMA may be associated with a second minus end–directed motor protein yet to be defined (Gaglio et al., 1996). This view is further supported by the observation that NuMA accumulated at spindle ends in the absence of dynein activity and that inhibition of NuMA blocked the movement of microtubule seeds on spindle arrays (Heald et al., 1997). Because NuMA has the ability to form filamentous networks, it has also been suggested that it may be mechanistically involved in the organization of microtubules of the mitotic spindle (Saredi et al., 1996) and/or serve to counterbalance the forces exerted against the microtubules by the putative motor to which it is attached (Gaglio et al., 1996).

The focus of the present study was to gain clues about the function of the novel 135-kD nonerythroid isoform of 4.1R by identifying its binding partners. We did not detect the presence of actin, spectrin, or calmodulin in the immunoprecipitates of nuclear or mitotic extracts, although these proteins are known to bind to 4.1R (Becker et al., 1990; Tanaka et al., 1991; Morris and Lux, 1995) in erythrocytes. It is possible that in association with 4.1R isoforms, these proteins serve different functions. Their association with nuclear 4.1 isoform(s), like the 135-kD 4.1R, has not been documented. Nevertheless, it appears that a nuclear isoform of 4.1R interacts with NuMA in the interphase nucleus. It is not yet known if 4.1R and NuMA remain associated throughout the cell cycle or if their association is dynamic. The biological significance of this interaction is also not known at this time. However, the data shown in Fig. 9 suggest that perturbations of protein 4.1R alter the localization of NuMA. Cells that overexpress the 135-kD 4.1R isoform perished within a time frame of three to four cell divisions. Even though our data do not define a particular role for 4.1R, it is tempting to speculate that 4.1R might stabilize the interaction among NuMA, dynein, dynactin, and microtubules, in a manner analogous to its role in stabilizing the association of spectrin, actin, and integral membrane proteins in red cells. This is further supported by the demonstration that 4.1 epitopes are localized in nuclei, and asters and mitotic spindles. Moreover, the fact that antibodies specific for 4.1 perturb the formation of these structures in the *Xenopus* system (Krauss, S.W., R. Heald, G. Lee, T. Berger, and J.A. Chasis. 1998. *Mol. Biol. Cell.* [Suppl.] 9:294a) lends additional support. However, more attempts to modify mitosis or in vitro reconstruction of astral arrays, by altering 4.1R expression or supply, will be required to test this notion.

The binding sites between 4.1R and NuMA have been mapped to the amino acids encoded by exons 20 and 21 of the COOH-terminal domain of 4.1R and to amino acids 1788–1810 of NuMA (Fig. 3). Because these residues of 4.1R are highly conserved among a growing 4.1R-like gene family (Walensky et al., 1998) it is possible that the polypeptide interacting with NuMA is a member of the 4.1R-like gene family. However, as discussed earlier, our results strongly implicate a 135-kD isoform of protein 4.1R. Of the three alternative COOH termini of NuMA that have been detected (Tang et al., 1993), amino acid residues 1788–1810 of NuMA, which are necessary for its

interaction with 4.1R, are located within an alternatively spliced exon that is also known to code for the COOH terminus required for its nuclear localization and spindle association. A comparison of human and frog amino acid residues of NuMA (Fig. 8 I) in the region required for its interaction with 4.1R shows that this sequence is highly conserved (95%), although the proteins have only 48% overall homology (Merdes et al., 1996). The especially strong conservation of the interacting sequences of NuMA and 4.1R (Huang et al., 1993) could reflect an important role in a fundamental cell process, such as cell division. Thus, it appears that the isoform of NuMA that localizes to the nucleus and associates with spindle also binds with 4.1R, and further supports our earlier contention that 4.1R is present in the nucleus (Marchesi, V.T., S. Huang, T.K. Tang, and E.J. Benz, Jr. 1990. *Blood*. 76:12a).

The precise role played by 4.1R in spindle assembly is not known at present. However, our data strongly suggest that 4.1R is involved in some fashion. A role for 4.1R in cell division is supported by data that meet the same criteria used to document the initial involvement of NuMA, dynein, and dynactin in this process. Since 4.1R forms a complex with all these proteins, and can alter the localization of NuMA when perturbed, our data suggest that 4.1R may also be crucial for the organization of the mitotic spindle. Further work will be needed to establish the steps in these processes for which protein 4.1R is important.

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