Protein 4.1R regulates interphase microtubule organization at the centrosome

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Summary

In human red blood cells, protein 4.1 (4.1R) stabilizes the spectrin-actin network and anchors it to the plasma membrane. To contribute to the characterization of functional roles of 4.1R in nonerythroid cells, we analysed the effect of ectopic expression of 4.1R isoforms on interphase microtubules in fibroblastic cells. We found that specific 4.1R isoforms disturbed the microtubule architecture but not the actin cytosskeleton. Biochemical sedimentation and/or confocal microscopy analyses showed that the pericentriolar components γ-tubulin and pericentrin remained at centrosomes, whereas the distributions of proteins p150Glued and the dynein intermediate chain were altered. Remarkably, 4.1R was displaced from the centrosome. In microtubule depolymerizing-repolymerizing assays, 4.1R-transfected cells showed an ability to depolymerize and nucleate microtubules that was similar to that of untransfected cells; however, microtubules became disorganized soon after regrowth. In microtubule-depolymerized transfected cells and during the initial steps of microtubule regrowth, centrosomal 4.1R localized with γ-tubulin but did not when microtubules became disorganized.

To learn more about centrosomal 4.1R function, isolated centrosomes were examined by confocal microscopy, western blot and in vitro microtubule aster-assembly assays. The experiments showed that 4.1R was present in isolated centrosome preparations, that it remained in the center of in-vitro-assembled microtubule asters and that more asters were assembled by the addition of protein 4.1R fused to glutathione-S-transferase. Together, these results indicate that 4.1R plays a key role at the centrosome, contributing to the maintenance of a radial microtubule organization.

Key words: Protein 4.1R, Centrosome, Microtubules

Introduction

Microtubule organization is essential for directional intracellular transport, the modulation of cell morphology and locomotion, and the formation of the spindle apparatus during cell division. In animal cells, the centrosome is the major microtubule-organizing center that, during interphase, originates a microtubule radial array. In fibroblasts, microtubules appear to project radially from a single spot, the microtubule-organizing center, implying that they remain tightly associated with the centrosome. By contrast, in neurons and polarized epithelial cells, many microtubules are released from centrosomes and become reorganized into nonradial arrays that project into neurites or away from the apical face of the cell (Dammermann et al., 2003; Doxsey, 2001). Centrosome-nucleated microtubules are polarized with their rapidly growing (plus) ends in the cytoplasm and their slowly growing (or minus) ends anchored at the centrosome. Cytoplasmic dynein is the predominant minus-end-directed microtubule motor in eukaryotic cells and usually conducts cargo in association with dynactin, a 20S complex consisting of at least nine polypeptides that appear to play an essential role in linking cargo to the dynein motor (Paschal et al., 1993). The dynein-dynactin complex has been shown to be a major contributor to microtubule organization and centrosome integrity (Quintyne et al., 1999).

Red-blood-cell protein 4.1 (4.1R or 4.1R80) was originally identified as an 80-kDa multifunctional protein of the membrane skeleton of human erythrocytes. In these cells, protein 4.1R stabilizes the spectrin-actin network and mediates the attachment of the underlying cytosskeleton to the overlaying lipid bilayer through interactions with integral membrane proteins (Conboy, 1993).

In nonerythroid cells, multiple isoforms of 4.1R are expressed as a result of extensive alternative splicing of the 4.1R-encoding pre-mRNA (Conboy, 1999; Tang et al., 1990). This event is cell and tissue specific, and also dependent on the growth and differentiation stages of the cell (Baklouti et al., 1997; Conboy et al., 1991; Chasis et al., 1993; Hou and Conboy, 2001; Schischmanoff et al., 1997). Immunological studies have detected 4.1R epitopes at different subcellular locations. Concomitantly, the association of 4.1R with proteins localized at different intracellular sites has been reported (Cohen et al., 1998; De Cárcer et al., 1995; Hou et al., 2000; Kontrogianni-Konstantopoulos et al., 2001; Kontrogianni-Konstantopoulos et al., 2000; Krauss et al., 1997; Lallena and Correas, 1997; Lallena et al., 1998; Mattagajasingh et al., 1997).
2000; Nunomura et al., 1997), thus suggesting that 4.1R might be involved in many processes in nucleated cells. A possible role for 4.1R in organizing the nuclear architecture is suggested by interactions observed between 4.1R and nuclear components of the splicing machinery (Lallena and Correas, 1997; Lallena et al., 1998). More recently, 4.1R has been shown to be essential for proper nuclear assembly (Krauss et al., 2002). A role has also been proposed for 4.1R in organizing microtubule architecture and the mitotic spindle poles as 4.1R interacts with interphase microtubules (Perez-Ferreiro et al., 2001), with a novel centrosomal protein termed CPAP (Hung et al., 2000), and with the nuclear mitotic apparatus protein (Mattagajasingh et al., 1999).

Proteins with key microtubule-anchoring functions are reported to provoke disorganization of the interphase microtubule array when ectopically expressed in COS-7 cells (Dammermann and Merdes, 2002; Quintyne et al., 1999). However, they might not affect interphase microtubule organization in other cell lines. Thus, dynamin, a component of the dynactin complex, was reported to have no effect on interphase microtubules in HeLa cells, whereas, conversely, COS-7 cells overexpressing dynamin contained microtubules that were less focused than normal (Quintyne et al., 1999). We have described the colocalization of ectopically expressed 4.1R with the microtubule network in human T cells, and the disruption of microtubule architecture in COS-7 cells (Perez-Ferreiro et al., 2001). To gain insights into the role of 4.1R in the organization of interphase microtubules, we have performed transfection experiments using different 4.1R-encoding cDNAs, microtubule depolymerization-repolymerization assays, confocal microscopy and biochemical sedimentation analyses in COS-7 cells. We have found that interphase microtubules are selectively perturbed by specific exogenous 4.1R isoforms and that microtubule disorganization is accompanied by altered distributions of the dynein-dynactin complex and, more interestingly, of centrosomal 4.1R. Results of further experiments indicate that 4.1R plays an essential role in regulating microtubule organization at the centrosome.

Materials and Methods

Cell culture and transfection

Human T-lymphoid Molt-4 and COS-7 cells were grown as described previously (Perez-Ferreiro et al., 2001). Transfection experiments were performed by electroporation using the Electro Cell Manipulator 600 (BTX, San Diego, CA). Cells were processed 48 hours after transfection.

cDNA cloning and composite cDNA constructs

The 4.1R135Δ4,5,16, 4.1R135Δ16, 4.1R135Δ16,19, 4.1R80, 4.1R80Δ16, 4.1R80Δ16,16 and 4.1R60Δ16,18 cDNAs were cloned from Molt-4 T cells and tagged as described (Luque and Correas, 2000; Luque et al., 1999). The 4.1R80Δ16,18-GFP cDNA was constructed as detailed elsewhere (Luque et al., 2003). The glutathione-S-transferase (GST), GST C-terminus (GST-Cter), GST-4.1R80Δ16,18 and GST-4.1R80Δ16 proteins were prepared as described elsewhere (Perez-Ferreiro et al., 2001).

Antibodies

Anti-c-Myc mouse monoclonal antibody 9E10 was obtained from the American Type Culture Collection. Anti-4.1R (10b) antibody was an affinity-purified polyclonal antibody generated as described previously (Correas et al., 1986). Anti-α-tubulin (DM1A), anti-γ-tubulin (GTU-88), anti-actin (AC-40) and anti-dynein (intermediate chain) mouse monoclonal antibodies were purchased from Sigma. Anti-tubulin antibody YL1/2 was a rat monoclonal antibody purchased from Sera-Lab. Anti-p150Glued antibody was a mouse monoclonal antibody from BD Transduction Laboratories. Anti-pericentrin antibody was a rabbit polyclonal antibody from Babco. Anti-GST antibody was a rabbit polyclonal antibody from Sigma. Horseradish-peroxidase-labeled secondary antibodies were obtained from Southern Biotechnology Associates. Fluorescence-labeled secondary antibodies and Alexa-Fluor-594/phalloidin were obtained from Molecular Probes.

Immunofluorescence and confocal microscopy

COS-7 cells were fixed with 4% formalin (37% formaldehyde solution, Sigma), permeabilized and blocked (De Cárcer et al., 1995). In some cases, cells were fixed with methanol-acetone (1:1 v/v) for 10 minutes at –20°C. Cells were incubated with the appropriate antibodies and processed as described (De Cárcer et al., 1995). Controls with primary antibodies omitted were included in each experiment. In figures 1-4, at least 100 transfected cells were analysed from four independent experiments. Images were obtained using a Zeiss epifluorescence microscope or a Bio-Rad Radiance 2000 confocal laser microscope.

Western-blot analysis

Protein samples were separated by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and transferred to Immobilon polyvinylidene difluoride (Millipore) in Tris-borate buffer, pH 8.2. Membranes were processed and developed as described (De Cárcer et al., 1995).

Microtubule regrowth assays

COS-7 cells were transfected, seeded on coverslips and grown for 48 hours as described above. Microtubules were depolymerized in 10 μM nocodazole (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM) for 4 hours and then washed three times with DMEM and incubated in fresh medium to allow microtubule regrowth. Coverslips were fixed at timed intervals and processed for immunofluorescence as described above.

Sedimentation assays

COS-7 cells were harvested, lysed and sedimented as described (Echeverri et al., 1996). Sedimentation standards, bovine serum albumin (4.4S), catalase (11.3S) and thyroglobulin (19S), were run in parallel. Equal volumes of each fraction were analysed by SDS-PAGE followed by western blotting (De Cárcer et al., 1995).

Centrosome isolation

Human centrosomes were isolated from Molt-4 T cells as previously described (Moudjou and Bornens, 1998). These cells were chosen because the centrosome isolation protocol used by Moudjou and Bornens (Moudjou and Bornens, 1998) is optimized for human T cells and gives a high yield. Briefly, nocodazole- and cytochalasin-D-treated Molt-4 T cells (~10⁶) were used for centrosome isolation. The centrosomal suspension obtained was layered onto a discontinuous sucrose gradient using the procedure described previously (Moudjou and Bornens, 1998). For immunofluorescence analysis, the centrosome-containing fractions were spun onto coverslips and probed with anti-α-tubulin, anti-γ-tubulin or anti-4.1R antibodies using previously described protocols (Moudjou and Bornens, 1998).
Microtubule nucleation assays

The microtubule-nucleation ability of isolated centrosomes was analysed according to the method of Mitchison and Kirchner (Mitchison and Kirchner, 1984). The centrosome-containing fractions were incubated in 80 mM PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid)], pH 6.8, 1 mM MgCl₂, 1 mM EGTA and 1 mM GTP; containing 3 mg ml⁻¹ bovine brain tubulin (Molecular Probes) for 8 minutes at 37°C. Microtubules were fixed by adding glutaraldehyde (1%), sedimented onto coverslips and then subjected to immunofluorescence analysis using anti-α-tubulin and anti-4.1R antibodies. 12 randomly chosen fields were examined on each coverslip under the microscope and the number of asters formed was counted. To test the effect of addition of GST-4.1R∆16,18, the isolated centrosomes were preincubated with the recombinant protein for 45 minutes at 4°C. GST alone was used as a negative control.

Pull-down assays

GST and the recombinant proteins GST-4.1R∆16,18 and GST-Cter were prepared as indicated (Perez-Ferreiro et al., 2001). COS-7 cell extracts were prepared by washing cells twice with PBS and adding lysis buffer (25 mM Tris, pH 7.6, 200 mM NaCl, 1% Triton X-100) containing 10 µg ml⁻¹ pepstatin, leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cells were scraped off the plate, transferred to Eppendorf tubes and incubated for 10 minutes on ice. The lysates were centrifuged at high speed in a minicentrifuge at 4°C for 10 minutes. The supernatants were recovered and incubated for 1 hour at 4°C with the glutathione/Seaphorase-4B column loaded with the corresponding GST proteins. After extensive washes with lysis buffer, the beads were resuspended in Laemmli buffer and boiled for 5 minutes. Recovery of the GST fusion proteins was confirmed by SDS-PAGE and Coomassie staining. The proteins were transferred to nitrocellulose membranes according to standard procedures and visualized by immunoblotting as described above.

Results

Perturbation of microtubule organization provoked by specific 4.1R isoforms exogenously expressed in COS-7 cells

We have previously shown that ectopic expression of protein 4.1R∆16 in COS-7 cells provoked interphase microtubule disorganization (Perez-Ferreiro et al., 2001). To gain insights into the role of protein 4.1R in the organization of interphase microtubules, we first examined whether microtubule perturbation is a general effect caused by ectopic expression of 4.1R in COS-7 cells. We chose to assay cDNAs encoding different 4.1R isoform types: isoforms translated from the most upstream start codon, ATG-1 (approximate molecular weight 135 kDa), and from the downstream start sites, ATG-2 (approximate molecular weight 80 kDa) and ATG-3 (approximate molecular weight 60 kDa) (Fig. 1A). The three 4.1R isoform types present different subcellular distribution: ATG1-translated 4.1R isoforms predominantly appear on non-nuclear sites (Luque et al., 1999); ATG2-translated 4.1R isoforms have a variable subcellular distribution (Gascard et al., 1998; Luque and Correas, 2000); and ATG3-translated 4.1R isoforms are predominantly located in the nucleus (Luque and Correas, 2000). Representative images are shown in Fig. 1B. More than 95% of the cells ectopically expressing the ATG1-translated isoform 4.1R∆16,18 predominantly showed organized microtubules radiating from the centrosome to the periphery (Fig. 1B, top, arrows). Ectopic expression of the ATG1-translated isoforms 4.1R∆16 and 4.1R∆16,19 was also tested, the results being similar to those for 4.1R∆16,18 (not shown). By contrast, disorganized, unfocused microtubules that no longer radiated from the centrosome were observed in most cells (~70%) expressing the ATG3-translated 4.1R isoform 4.1R∆16,18 (Fig. 1B, middle, arrows). An examination of the consequences of ectopic expression of the ATG2-translated 4.1R isoforms 4.1RΔ80, 4.1RΔ16 and 4.1RΔ5,16 yielded heterogeneous results whereby some isoforms affected microtubule organization...
more than others (40%, 33% and 45%, respectively). This effect correlated well with the subcellular localization of the expressed ATG2-translated 4.1R isoform (Fig. 1B, bottom), which, as previously described, is variable (Gascard et al., 1998; Luque and Correas, 2000).

These results indicate that: (1) perturbation of the microtubule cytoskeleton is not a general effect provoked by the ectopic expression of 4.1R in COS-7 cells; and (2) this perturbation seems to correlate with nuclear localization of the expressed 4.1R isoforms, suggesting that 4.1R would need to be compartmentalized to induce this effect. To learn more about the basis of the microtubule disorganization observed, we used the 4.1R60∆16,18 protein in this study, because this was the 4.1R isoform that had the strongest effect.

Actin cytoskeleton is not affected by ectopic expression of protein 4.1R60∆16,18

We next investigated whether another component of the cytoskeleton, actin, was also perturbed by the ectopic expression of 4.1R60∆16,18. Untransfected cells possessed focused microtubules radiating from the centrosome and actin was detected in stress fibers and membrane ruffles (Fig. 1C). In the same image, cells expressing 4.1R60∆16,18 showed unfocused microtubules (Fig. 1C, tubulin, arrows) but no evidence of perturbation in actin distribution was observed (Fig. 1C, actin, arrows). These results were observed in approximately 98% of the transfected cells and indicate that ectopic expression of 4.1R60∆16,18 perturbs microtubule but not actin organization.

γ-Tubulin and pericentrin distributions are not affected by ectopic expression of protein 4.1R60∆16,18

We next sought to establish whether the distribution of two main centrosomal components involved in microtubule nucleation, γ-tubulin and pericentrin, were altered in cells presenting disorganized microtubules. In control cells, microtubules were well organized and γ-tubulin and pericentrin localized to a single focus or paired foci near the nucleus (Fig. 2, asterisks). In the same image, cells expressing protein 4.1R60∆16,18 (Fig. 2, left, arrows), had disorganized microtubules (Fig. 2, middle, arrows), whereas the staining patterns for γ-tubulin and pericentrin (Fig. 2, right, arrows) were similar to those in control cells. These results were observed in ∼95% of the transfected cells.

Dynactin and dynein distributions are affected by ectopic expression of protein 4.1R60∆16,18

One possible explanation for the microtubule disorganization observed in cells ectopically expressing protein 4.1R60∆16,18 might be that the localization of some essential component(s) for stabilizing microtubule organization at centrosomes could be affected. Protein p150Glued is one of the subunits of the dynactin complex that appears to perform a key microtubule-anchoring function (Quintyne et al., 1999). We therefore investigated whether cells overexpressing protein 4.1R60∆16,18 have a normal or altered p150Glued distribution. Untransfected control cells presented a single focus or paired foci near the nucleus and cytoplasmic staining (Fig. 3A, p150Glued, asterisks) whereas cells expressing protein 4.1R60∆16,18 showed either p150Glued distributed in several foci dispersed throughout the cytoplasm and cytoplasmic staining (Fig. 3A, p150Glued, top, arrow) or no detectable p150Glued fluorescence staining at the centrosome (Fig. 3A, middle, insets).

Dynein intermediate chain (IC) mediates the dynein-dynactin interaction through association with p150Glued and is required for interphase microtubule organization at centrosomes (Vaughan and Vallee, 1995). We were therefore interested to establish whether cells ectopically expressing protein 4.1R60∆16,18 have normal or altered dynein distribution. Untransfected control cells presented a single focus or paired foci near the nucleus (Fig. 3A, dynein, bottom, asterisks) whereas cells expressing protein 4.1R60∆16,18...
showed, as in the case of protein p150Glued, either several foci dispersed throughout the cytoplasm (not shown) or no detectable dynein fluorescence staining at the centrosome (Fig. 3A, bottom, insets). The effects observed for dynein and p150Glued distributions are representative of most transfected cells (~70%).
Overexpression of 4.1R<sub>60Δ16,18</sub> had similar effects on microtubule organization to those caused by some dynactin components, so we set out to determine whether interphase cells had disrupted dynactin structure (Echeverri et al., 1996; Quintyne et al., 1999). To address this matter, we examined whether dynactin-dynein remained a single complex that sedimented at ~20S, as described by others (Paschal et al., 1993). In agreement with previous results from control COS-7 cells (Echeverri et al., 1996), p150<sub>Glued</sub> and dynein migrated as an ~18-19S and an ~20S complex, respectively (Fig. 3B, Ct). In samples prepared from cells ectopically expressing 4.1R<sub>60Δ16,18</sub>, we observed no major alterations in dynactin or dynein sedimentation behaviors. We did notice, however, that a small proportion of p150<sub>Glued</sub> and dynein fractionated with sedimentation coefficients higher than 19S or 20S, respectively, in fraction 1 (Fig. 3B, Tr). It is plausible that p150<sub>Glued</sub> and dynein detected in fraction 1 represent that isolated from the small cell population ectopically expressing 4.1R<sub>60Δ16,18</sub> (~10% of total cells) and are therefore representative of the altered localization observed for these proteins in the confocal images (Fig. 3A). Interestingly, fraction 1 also contained endogenous 4.1R proteins of ~135-140 kDa that were absent from fraction 1 isolated from control cells (Fig. 3B, bottom). This result indicates that the sedimentation behavior of endogenous protein 4.1R, like p150<sub>Glued</sub> and dynein, was also affected by the ectopic expression of 4.1R<sub>60Δ16,18</sub>.

**Fig. 4.** Effects of exogenous 4.1R expression on microtubule nucleation and retention at the centrosome. Cells were treated with nocodazole to promote microtubule disassembly and then washed to allow microtubule regrowth. At the indicated times, untransfected (A) and transfected (B) cells were fixed and triple labeled with the following antibodies: YL1/2 to detect microtubules (tubulin); GTU-88 to detect γ-tubulin (γ-tubulin); and 10b to detect endogenous and exogenous 4.1R (4.1R). Notice that, in cells with disorganized microtubules, γ-tubulin remains at the centrosome, whereas centrosomal 4.1R does not. The images of untransfected (A) and transfected (B) cells were taken from the same field. The images are projections of optical-section stacks acquired by confocal microscopy and insets are enlargements showing the distribution of each protein at the centrosome. Bar, 20 µm.

4.1R<sub>60Δ16,18</sub> was also perturbed by ectopic expression of protein 4.1R<sub>60Δ16,18</sub> protein. 4.1R has been detected at the centrosome in interphase cells (Krauss et al., 1997) and associated with the centrosomal protein CPAP (Hung et al., 2000). It seemed plausible that centrosomal 4.1R distribution could be altered in cells showing disorganized microtubules by ectopically expressing 4.1R<sub>60Δ16,18</sub>. To test this hypothesis, control cells and cells overexpressing protein 4.1R<sub>60Δ16,18</sub> were triple stained with antibodies GTU-88 (to identify γ-tubulin), YL1/2 (to detect tubulin) and 10b (to identify simultaneously the endogenous centrosomal 4.1R and the exogenously expressed 4.1R isoform). Several confocal images were analysed and the section in which the centrosome was identified with the anti-γ-tubulin antibodies was carefully compared with the staining.
obtained for 4.1R. Most untransfected control cells (>96%) showed a normal microtubule cytoskeleton as detected with anti-tubulin antibody, and a brightly stained centrosome as detected with anti-γ-tubulin and anti-α-tubulin (α-tubulin) antibodies (Fig. 3C, asterisks). Most cells ectopically expressing 4.1R60Δ16,18 (∼70%) contained disorganized interphase microtubules and a brightly stained centrosome as detected with anti-γ-tubulin antibody, but had no detectable (Fig. 3C, insets) or highly reduced (not shown) 4.1R fluorescence staining at the centrosome. These results indicate that exogenous 4.1R alters the microtubule architecture and also affects endogenous centrosomal 4.1R distribution.

Effects of 4.1R60Δ16,18 overexpression on microtubule nucleation and organization at centrosomes

Centrosome functions such as microtubule nucleation and the organization of a radial microtubule network were examined and correlated with the distribution of centrosomal 4.1R in these processes. Microtubules were depolymerized using nocodazole and the subsequent pattern of microtubule regrowth was determined (Fig. 4). After increasing intervals of regrowth (0 minutes to 3 hours), cells were fixed and triple stained to detect tubulin, γ-tubulin and 4.1R, and confocal images were analysed. Untransfected cells (Fig. 4A) initially had depolymerized microtubules (Fig. 4A,α-tubulin, 0'). After 5 minutes of regrowth, cells showed single microtubule asters and endogenous centrosomal 4.1R distribution was unaltered (5'; compare 4.1R with γ-tubulin). By 30 minutes of regrowth, microtubules and endogenous centrosomal 4.1R distributions differed notably from those of control cells. Thus, disorganized, unfocused microtubules were detected (Fig. 4B, tubulin, 30'), accompanied by a lack of endogenous centrosomal 4.1R fluorescence staining (Fig. 4B,4.1R, 30') but with an unaltered γ-tubulin distribution (Fig. 4B,γ-tubulin, 30'). The distribution of exogenous nuclear 4.1R60Δ16,18 is also shown in Fig. 4B. Exogenous 4.1R did not contribute to the observed centrosomal staining because the anti-tag antibody did not stain the centrosomes of transfected cells (not shown; see also Fig. 1). The results of these experiments imply that transfected cells could depolymerize microtubules correctly and nucleate them properly from the centrosome, but that the ability of the centrosome to retain newly assembled microtubules was disturbed. Interestingly, the correct microtubule depolymerization and nucleation from the centrosome correlated with the presence of 4.1R at the centrosome, whereas the inability of the centrosome to anchor microtubules and maintain a radial microtubule organization correlated with an altered distribution of centrosomal 4.1R.

Endogenous protein 4.1R is present in isolated centrosome preparations and localizes within the center of microtubule asters assembled in vitro

Although protein 4.1R was recently localized to the centrosome by immunofluorescence and electron microscopy of mammalian tissue culture lines (Krauss et al., 1997), the presence of 4.1R was not detected in either biochemical experiments or immunofluorescence assays in isolated centrosome preparations (Hung et al., 2000). To learn more about centrosomal 4.1R, we isolated centrosomes on sucrose
We next analysed the effect that addition of GST-4.1R60 protein GST-4.1R60 on microtubule aster formation. The isolated centrosomes were preincubated with buffer (Ct), GST (GST), GST-4.1R80 Δ16 (GST-4.1R80) or GST-4.1R60 Δ16,18 (GST-4.1R60) for 45 minutes at 4°C and then tubulin was added to start in-vitro microtubule-aster formation. The microtubule asters stained with anti-α-tubulin antibody are shown (red). A representative aster (boxed field in the GST-4.1R60 panel) double stained with anti-α-tubulin (red) and anti-GST (green) antibodies is shown in the enlargements. The recombinant GST-4.1R60 protein is incorporated into the center of the asters and along the microtubules (merge). Four experiments were performed in duplicate. The results are shown in the histogram as the mean ± the standard deviation of the number of microtubule asters assembled in vitro. The images are projections of optical-section stacks acquired by confocal microscopy. Bar, 10 μm.

**Fig. 6.** Effect of recombinant protein GST-4.1R60 Δ16,18 on microtubule aster formation. The isolated centrosomes were preincubated with buffer (Ct), GST (GST), GST-4.1R80 Δ16 (GST-4.1R80) or GST-4.1R60 Δ16,18 (GST-4.1R60) for 45 minutes at 4°C and then tubulin was added to start in-vitro microtubule-aster formation. The microtubule asters stained with anti-α-tubulin antibody are shown (red). A representative aster (boxed field in the GST-4.1R60 panel) double stained with anti-α-tubulin (red) and anti-GST (green) antibodies is shown in the enlargements. The recombinant GST-4.1R60 protein is incorporated into the center of the asters and along the microtubules (merge). Four experiments were performed in duplicate. The results are shown in the histogram as the mean ± the standard deviation of the number of microtubule asters assembled in vitro. The images are projections of optical-section stacks acquired by confocal microscopy. Bar, 10 μm.

**Protein 4.1R associates with γ-tubulin and α-tubulin**

The results obtained above suggested that protein 4.1R might function as a microtubule-anchoring factor, establishing a bridge between microtubules and the centrosome. We performed pull-down assays to determine whether 4.1R associates with the centrosomal and microtubule components γ- and α-tubulin, respectively. Glutathione-Sepharose beads coupled with GST-4.1R60 Δ16,18, GST-Cter or GST were used on COS-7 cell extracts. As shown in Fig. 7, GST-4.1R60 Δ16,18, but not GST-Cter or GST control beads, copelleted γ- and α-tubulin from the cell extracts, indicating that 4.1R has the ability to associate with γ- and α-tubulin through a region other than its C-terminus.

**Discussion**

Many studies have attempted to determine the mechanisms and proteins involved in focusing microtubule minus ends into the spindle poles. However, there is less information about the mechanisms and proteins regulating microtubule organization at the centrosome in interphase cells. The present report yields new insights into the function of 4.1R in nonerythroid cells by showing that 4.1R plays a key role at the centrosome, contributing to the maintenance of a radial microtubule organization.

Although protein 4.1R was recently localized to the centrosome by immunofluorescence and electron microscopy of mammalian tissue culture lines (Krauss et al., 1997), the presence of 4.1R was not detected in either biochemical experiments or immunofluorescence assays in isolated centrosome preparations (Hung et al., 2000). Our analyses using isolated centrosomes showed that 4.1R was present at the centrosome, that 4.1R localized at the central part of microtubule asters assembled in vitro and that addition of GST-4.1R60 Δ16,18 (GST-4.1R60) enhanced the number of asters

...density gradients following the procedure described by Modjou and Bornens (Moudjou and Bornens, 1998). Fig. 5A shows representative images of isolated centrosomes double stained with anti-4.1R and anti-γ-tubulin or anti-α-tubulin antibodies. As shown in Fig. 5A, protein 4.1R was present in the isolated centrosome fractions. Western-blot analysis of isolated centrosomes showed 4.1R major bands of ~135 kDa and ~80 kDa and a minor ~60 kDa band (Fig. 5B, 4.1R). γ-Tubulin was present in the isolated centrosome preparations whereas actin was absent (Fig. 5B, γ-tubulin and actin). It is not certain whether the centrosomal 4.1R band of ~135 kDa corresponds to that abnormally sedimenting with dynein and p150Glued in fraction 1 (Fig. 3B) and that is therefore undetectable at the centrosome of cells exogenously expressing 4.1R60 (Fig. 3C).

The isolated centrosomes were tested for their ability to nucleate and assemble microtubule asters in vitro and the location of 4.1R within the asters was determined by confocal microscopy. Most centrosomes (~80%) had the ability to assemble in vitro microtubule asters and protein 4.1R, like γ-tubulin, was localized at the central part of the microtubule aster (Fig. 5C).

**Effect of recombinant GST-4.1R on microtubule aster formation**

We next analysed the effect that addition of GST-4.1R60 Δ16,18 (GST-4.1R60) has on microtubule aster formation. Recombinant GST-4.1R60 Δ16 protein (GST-4.1R60) was also used in the assay because 4.1R60 Δ16, unlike 4.1R80 Δ16,18, distributed predominantly to the cytoplasm of cells (Luque and Correas, 2000). The centrosomes were preincubated in the absence (control sample, Ct) or the presence of GST, GST-4.1R80 or GST-4.1R60 and were then assayed for aster formation by the addition of tubulin. Addition of GST had no apparent effect on microtubule aster formation compared with control samples. By contrast, addition of GST-4.1R60 increased the number of total microtubule asters assembled in vitro (Fig. 6). The recombinant protein GST-4.1R60 was incorporated at the center of the asters and along the microtubules (Fig. 6, small panels).
Proteins with key microtubule-anchoring functions are reported to provoke disorganization of the interphase microtubule array when ectopically expressed in COS-7 cells (Dammermann and Merdes, 2002; Quintyne et al., 1999). The significance of dynein and dynactin for microtubule anchoring and their participation in the regulation and turnover of the microtubule network are clearly important (Bornens, 2002; Dammermann et al., 2003). Indeed, when components of the dynactin complex were overexpressed in COS-7 cells, interphase microtubules were not focused into radial arrays and dynactin subunits did not accumulate at microtubule minus ends (Burkhardt et al., 1997; Clark and Meyer, 1999; Quintyne et al., 1999). Using the same type of experiments, our results showed that specific 4.1R isoforms provoked similar perturbation of the interphase microtubule organization and that, importantly, 4.1R was displaced from the centrosome. These results, in conjunction with the finding that endogenous 4.1R proteins and a population of p150Glued and dynein proteins fractionated on sucrose density gradients with altered sedimentation coefficients (>20S), suggested that microtubule organization is profoundly influenced by the activity and subcellular localization of protein 4.1R.

Ninein, centrin and PCM1 are microtubule-anchoring factors that require dynein/dynactin motor function for their recruitment to the centrosome (Dammermann and Merdes, 2002). Depletion of any of these proteins provoked loss of centrosomal microtubule organization (Dammermann and Merdes, 2002). As for PCM1 depletion, the absence of centrosomal 4.1R was accompanied by centrosomal microtubule disorganization, altered distributions of dynein and p150Glued and unaltered distribution of γ-tubulin. By contrast, unaltered distribution of centrosomal 4.1R correlated well with a correct microtubule depolymerization, nucleation and radial organization. These results indicate that, during interphase, 4.1R is important for the anchoring of microtubules at the centrosome and for the maintenance of radial arrays of microtubules.

Most of the data presented in this work were produced using isoform 4.1R60Δ16,18. The 4.1R60Δ16,18-encoding cDNA was originally isolated from Molt-4 T cells (Luque and Correas, 2000) and, interestingly, endogenous 4.1R proteins of ~60 kDa have also been detected in many other cell types (Luque and Correas, 2000; Anderson et al., 1988). Whether any of these endogenous 4.1R proteins correspond to isoform 4.1R60Δ16,18 is not known.

The ATG3-translated isoform 4.1R60Δ16,18 had the strongest effect on microtubule organization and presented a predominant nuclear localization. By contrast, ATG1-translated 4.1R isoforms had no effect and presented non-nuclear distribution. ATG2-translated 4.1R isoforms provoked microtubule disorganization if they had a predominant nuclear expression (Fig. 1B, bottom). From these results, it could be suggested that overexpression of 4.1R isoforms with nuclear localization could provoke the dramatic phenotype we show with isoform 4.1R60Δ16,18. The mechanism by which exogenous expression of nuclear 4.1R might affect microtubule organization is not yet established. One possible explanation could be that the overexpressed nuclear 4.1R isoform retained centrosomal 4.1R and/or a cytoplasmic component essential for maintaining a correct microtubule organization.

In summary, the functional analysis using isolated centrosomes allowed us to analyse the effect on microtubule aster assembly of adding GST-4.1R, whereas the 4.1R-overexpression assays were a useful tool to examine centrosomes lacking endogenous 4.1R. A plausible interpretation of our results is that centrosomal protein 4.1R could be an anchoring protein that establishes a bridge between the microtubules and the centrosome, hence participating in the
dynamic interrelationships between the centrosome and the cytoarchitecture.

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