Evidence for a Role of CLIP-170 in the Establishment of Metaphase Chromosome Alignment

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Abstract. CLIPs (cytoplasmic linker proteins) are a class of proteins believed to mediate the initial, static interaction of organelles with microtubules. CLIP-170, the CLIP best characterized to date, is required for in vitro binding of endocytic transport vesicles to microtubules. We report here that CLIP-170 transiently associates with prometaphase chromosome kinetochores and codistributes with dynein and dynactin at kinetochores, but not polar regions, during mitosis. Like dynein and dynactin, a fraction of the total CLIP-170 pool can be detected on kinetochores of unattached chromosomes but not on those that have become aligned at the metaphase plate. The COOH-terminal domain of CLIP-170, when transiently overexpressed, localizes to kinetochores and causes endogenous full-length CLIP-170 to be lost from the kinetochores, resulting in a delay in prometaphase. Overexpression of the dynactin subunit, dynamitin, strongly reduces the amount of CLIP-170 at kinetochores suggesting that CLIP-170 targeting may involve the dynein/dynactin complex. Thus, CLIP-170 may be a linker for cargo in mitosis as well as interphase. However, dynein and dynactin staining at kinetochores are unaffected by this treatment and further overexpression studies indicate that neither CLIP-170 nor dynein and dynactin are required for the formation of kinetochore fibers. Nevertheless, these results strongly suggest that CLIP-170 contributes in some way to kinetochore function in vivo.

Microtubules (MTs)[1] in vertebrate somatic cells are involved in intracellular transport and distribution of membranous organelles. Fundamental to this role are their tightly controlled, polarized organization, and unusual dynamic properties (Hirokawa, 1994) and their interaction with a complex set of MT-based motor proteins (Hirokawa, 1996; Sheetz, 1996; Goodson et al., 1997). During mitosis, they contribute to the motility of centrosomes, the construction of spindle poles (Karsenti et al., 1996; Merdes and Cleveland, 1997), and the dynamic movements of kinetochores (Rieder and Salmon, 1994) and chromosome arms (Barton and Goldstein, 1996; Vernos and Karsenti, 1996). The motor protein cytoplasmic dynein, drives the transport toward MT minus-ends of a variety of subcellular organelles (Schnapp and Reese, 1989; Schroer et al., 1989; Holzbaur and Vallee, 1994). Dynactin is a molecular complex originally identified as being essential for dynein-mediated movement of salt-washed vesicles in vitro (reviewed in Schroer, 1996; Schroer and Sheetz, 1991). Genetic studies in fungi, yeast, and flies have shown that the two complexes function together to drive nuclear migration, spindle and nuclear positioning and to permit proper neuronal development (Eshel et al., 1993; Clark and Meyer, 1994; Muhua et al., 1994; Plamann et al., 1994; McGrail et al., 1995; Karsenti et al., 1996). Biochemical studies suggest a direct interaction between certain subunits of dynein and dynactin (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). In vivo, the two molecules may bind one another transiently, since they have not been isolated as a stable complex.

There is good evidence indicating that the dynein/dynactin complex, together with other motors (Eg5, and a minus-end oriented kinesin-related protein) and a structural protein (NuMa), drive the focusing of free microtubule ends into mitotic spindle poles (Merdes and Cleveland, 1997; Waters and Salmon, 1997). A trimolecular complex composed of NuMa and dynein/dynactin may be crucial in this process in both acentriolar (Merdes et al., 1996), and centriolar spindles (Gaglio et al., 1997). A number of findings also indicate that the combined actions of dynein and dynactin at the kinetochore contribute to chromosome alignment in vertebrate somatic cells. First, the initial interaction between polar spindle MTs and kineto-

1. *Abbreviations used in this paper*: CLIP, cytoplasmic linker protein; MT, microtubules.

chores seems to involve a tangential capture event (Merdes and De Mey, 1990; Rieder and Alexander, 1990) which is followed by a poleward gliding along the surface lattice of the MT (Hayden et al., 1990). Both in vivo and in vitro (Hyman and Mitchison, 1991) this gliding movement appears similar to the dynein-mediated retrograde transport of vesicular organelles along MTs. Consistent with this is the finding that both dynein (Pfarr et al., 1990; Steuer et al., 1990) and its activator, dynactin (Echeverri et al., 1996), are present at prometaphase kinetochores. Overexpression of dynamitin, a 50-kD subunit of the dynactin complex, results in the partial disruption of the dynactin complex and in the loss, from kinetochores, of dynein, as well as dynactin. Therefore, it has been proposed that dynactin mediates the association of dynein with kinetochores. Abnormal spindles with poorly focused poles are observed and the cells become arrested in pseudoprometaphase (Echeverri et al., 1996). Despite these findings, rigorous proof for a role of the dynein motor complex in kinetochore motility is still lacking, and its role may differ between lower and higher eucaryotes, and between mitosis and meiosis.

CLIP-170 (Rickard and Kreis, 1996) is needed for in vitro binding of endocytic transport vesicles to MTs (Pierre et al., 1992). It is a nonmotor MT-binding protein that accumulates preferentially in the vicinity of MT plus ends and on early endosomes and endocytic transport vesicles in nondividing cells (Rickard and Kreis, 1990; Pierre et al., 1992). Like many MT-binding proteins, CLIP-170 is a homodimer whose NH_2-terminal head domains and COOH-terminal tail domains flank a central α-helical coiled-coil domain. The binding of CLIP-170 to MTs involves a 57–amino acid sequence present twice in the head domain (Pierre et al., 1992) and is regulated by phosphorylation (Rickard and Kreis, 1991). The COOH-terminal domain has been proposed to participate in targeting to endocytic membranes (Pierre et al., 1994). The fact that the latter move predominantly toward microtubule minus ends in a process most likely mediated by cytoplasmic dynein and dynactin (Aniento and Gruenberg, 1995), suggests that CLIP-170 may act in concert with this motor complex, and may be subject to regulated interactions with one or more dynactin or dynein subunits at the vesicle membrane.

Here we report that during mitosis, CLIP-170 codistributes with dynein and dynactin at kinetochores, but not spindle poles. Evidence is presented that the COOH-terminal domain of CLIP-170 is responsible for its kinetochore targeting, and that this may be mediated by the complex of dynein and dynactin. The effects on mitotic progression of overexpression of wild type and several deletion mutants of CLIP-170 provide evidence for the involvement of CLIP-170 in kinetochore function early in mitosis. We also present in vivo evidence that neither CLIP-170 nor the complex of dynein and dynactin are required for formation of kinetochore fibers.

Materials and Methods

Cell Culture and Drug Treatments

HeLa, A431, COS-7, and Caco-2 cells (all from the American Type Culture Collection, Rockville, MD) were grown, respectively, in MEM, RPMI, and DMEM (Caco-2 and COS-7), all supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% FCS (all reagents from Sigma, France) at 37°C in 5% CO_2. COS-7 cells medium included 1% nonessential amino acids. For immunolocalization, cells were plated on 14×14-mm glass coverslips in six-well dishes and grown for 24–48 h to reach 70–80% confluency. HeLa S3 cells were grown in suspension. For drug treatment experiments, cells were incubated either for 4 h with 10 μM vinblastine or for 1 h with 33 μM nocodazole (Sigma), before fixation.

Construction of Expression Plasmids and Transfections

All versions of CLIP-170 used in this study were fusion proteins tagged with the c-myc epitope (Evan et al., 1985) at the amino terminus. The plasmids encoding wild-type CLIP-170, CLIP-170 Δ55-346 (lacking the MT-binding regions), and CLIP-170 Δ1240-1392 (a mutant lacking the 152 COOH-terminal residues which includes the entire COOH-terminal domain and the COOH-terminal portion of the coiled coil), are referred to in the present paper as wt-CLIP-170, ΔN, and ΔC, respectively, and have been described elsewhere (Pierre et al., 1994). The plasmids were gifts of Drs. T. Kreis and P. Pierre (University of Geneva, Switzerland). Their expression is under control of the CMV promotor of the pcDNA1 vector (Invitrogen, San Diego, CA). ΔNΔC, a deletion construct that combines the deletions in ΔN and ΔC, was created by deleting the COOH terminus of ΔN as described for the construct ΔC1240. Expression of CLIP-170 ΔNΔC is under control of the SV-40 promotor of the pSG5 vector (Green et al., 1988). CLIP-170 ΔNΔR, lacking the MT-binding regions (ΔN) and most of the central coiled-coil rod (R) domain (amino acids 391–1230) is carried by the pcDNA1 vector. ΔNΔR was created by NheI–XbaI digestion of ΔN and subsequent ligation. Chicken dynamitin (p50), carried by the GW1-CMV vector, is as described (Gaglio et al., 1996).

For transient transfections, COS-7 cells were seeded onto 14×14-mm coverslips at $1–3 \times 10^5$ cells per well and grown for 24 h. 7–10 μg of plasmid DNA purified on Qiagen columns (Qiagen GmbH, Düsseldorf, Germany) was used in standard calcium phosphate (Sambrook et al., 1989) transfections. Transfected cultures were fixed 30–45 h later.

For intranuclear microinjection of plasmids, A431 cells were seeded at 75% confluency in medium containing 2 μg/ml aphidicolin (Sigma) on marked coverslips (CELLocate; Eppendorf, Hamburg, Germany). After 24 h the medium was replaced with normal medium. 4 h later the cells were transferred to medium containing 10 mM Hepes and microinjected as previously reported (Compton and Cleveland, 1993) using a semiautomatic microinjector and sterile Femtotip needles (Eppendorf). 2 h after microinjection, cells were incubated in medium containing nocodazole (33 μM) for 1 h, and fixed as described below. The overexpressing cells were relocated within the grid pattern on the CELLocate coverslips on the basis of their coordinates and the immunofluorescent signal.[1]

Chromosome Isolation

Chromosomes isolated from mitotic HeLa S3 cells after colcemid arrest according to the protocol for isolation of polyamine chromosomes (Gasser and Laemmli, 1987) were a gift of Dr. U. Laemmli (University of Geneva, Switzerland).

Preparation of Cell and Chromosome Fractions for SDS-PAGE

HeLa S3 cells grown on 500-cm^2 plastic dishes (Nunc) were washed with PBS and PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM Mg-acetate, pH 6.9), scraped into a minimal volume of ice cold PHEM using a rubber policeman and pelleted for 10 min in a Biofuge A (Heraeus, Osterode, Germany) at 13,000 rpm. Cells were resuspended in a volume equal to that of the pellet of PHEM, containing 1 mM PMSF, 2 mM DTT, and 40 μg/ml of cytochalasin D, aprotinin, leupeptin, and pepstatin A (all from Sigma). Cells were then lysed in twice this volume of hot (95°C) Laemmli sample buffer (Laemmli, 1970), centrifuged for 30 min at 4°C, 200,000 g, and the supernatants were used for SDS-PAGE. Isolated chromosomes were dissolved in hot sample buffer and centrifuged as above.

Immunological Methods

Two antibodies against CLIP-170 were used predominantly in this work: monoclonal antibody 4D3 (Rickard and Kreis, 1991) and rα55, an affinity-purified polyclonal antibody against a bacterially expressed fusion protein

(Pierre et al., 1992). mAb 4D3 reacts with the COOH-terminal portion of the coiled-coil rod domain that is deleted from the ΔNΔR mutant, but is present in all the others. A third antibody against CLIP-170, H2A, was raised by us in a rabbit against a His-tagged fusion protein comprising the 485 NH2-terminal amino acids of CLIP-170 (gift of Dr. T. Kreis). As seen by immunofluorescence of expressing cells, H2A recognizes all the CLIP-170 species studied here, probably because they all contain a short NH2-terminal sequence. pAb H2A and mAb 4D3 were used together on ΔNΔR overexpressing cells in order to mark, respectively, expressing cells (H2A) and endogenous CLIP-170 (4D3, which does not recognize ΔNΔR). Other antibodies were A27, a polyclonal anti-Arp1 (gift from Dr. D. Meyer, UCLA, Los Angeles, CA), monoclonal anti-p150^{GLUED} and a polyclonal anti-dynamitin, mAb 74.1, monoclonal anti-cytoplasmic dynein intermediate chain (gift from Dr. K. Pfister, University of Virginia, Charlottesville, VA), human CREST autoimmune anti-serum recognizing the centromeric region of chromosomes (gift from Dr. H. Ponstingl, German Cancer Research Center, Heidelberg, FRG), monoclonal anti-β-tubulin (Zymed Laboratories, Inc., South San Francisco, CA), monoclonal anti-myc (9E10; Evan et al., 1985; gift from Dr. T. Kreis, University of Geneva, Switzerland).

For Western blotting, samples were separated by SDS-PAGE, and transferred electrophoretically to a nitrocellulose membrane (Schleicher and Schuell Co., Dassel, Germany). Immunolabeling was done as described (Rickard and Kreis, 1990) except that antibodies were detected using enhanced chemiluminescence according to the supplier's directions (ECL, Amersham Corp., Arlington Heights, IL).

For double staining of A431 cells with polyclonal or monoclonal anti–CLIP-170 and human anti-CREST, the cells were fixed for 20 min in 3% paraformaldehyde in PHEM, washed three times 5 min in PBS and permeabilized for 25 min in 0.5% Triton X-100 in PBS. For triple staining of A431 cells with polyclonal anti-Arp1, monoclonal anti–CLIP-170 and human anti-CREST in experiments aiming at studying the relative level of kinetochore binding of these two proteins, the cells were fixed in the same way, but permeabilized for 4 min by incubation in methanol at −20°C. This fixation schedule was the only one allowing for optimal detection of Arp1 and CLIP-170 at the same time. For triple staining using anti-p150^{GLUED} or anti-dynein intermediate chain instead of Arp1, the cells were fixed for 6 min in methanol at −20°C alone. Because with this fixation schedule, the anti–CLIP-170 antibodies do not react very well with their antigen, they were used at a lower dilution, resulting in a higher background.

For determining the kinetochore-targeting domain of CLIP-170, transfected COS-7, and A431 cells were labeled for expressed CLIP-170 forms and CREST antigens. They were quickly rinsed in PHEM, lysed for 30 s in 0.5% Triton X-100 in PHEM to remove excess soluble expressed protein, fixed for 20 min in 3% paraformaldehyde (from a 16% stock in H2O) in PHEM at room temperature, washed three times 5 min in PBS and further permeabilized for 25 min in 0.5% Triton X-100 in PBS. The prelysis step leads to a significant loss of mitotic cells in COS-7 cells and also yields very reduced cytoplasmic signals in expressing cells. This made positive identification of these cells more difficult, and nearly impossible in the case of nonbinding CLIP-170 species. The procedure using A431 cells as outlined above was therefore used to confirm the absence of kinetochore binding of certain CLIP-170 forms.

To determine the mitotic phase indexes and spindle morphology of COS-7 cells overexpressing the various constructs, the primary fixation consisted of an incubation of exactly 12 min in 4% paraformaldehyde, 0.05% glutaraldehyde (EM-grade; Sigma), and 0.05% Triton X-100 at room temperature. The cells were then triple labeled for the expressed CLIP-170 form or dynamitin plus tubulin and DNA. This procedure yielded optimal spindle MT preservation. A similar procedure, but using a 4% paraformaldehyde, 0.01% glutaraldehyde, and 0.05% Triton X-100 fixation for 12 min was used to study the formation of kinetochore fibers in overexpressing COS-7 cells, by triple labeling overexpressed forms of CLIP-170 or dynamitin, tubulin and centromeric regions. This low concentration of glutaraldehyde significantly improved spindle MT preservation over formaldehyde alone, without suppressing the reactivity of the fixation sensitive CREST antigens. In the above experiments, the cells were not prelysed, since we needed the signal from the expressed protein to evaluate its relative level of expression in each cell.

Glutaraldehyde fixations were followed by two 10 min incubations in 10 mg/ml NaBH4 in PBS, pH 8.0. After a rinse in PBS and two 10 min washes in PBS/BSA, (PBS containing 0.05% BSA), the coverslips were incubated with primary antibodies for 1 h at 37°C, washed three times for 10 min with PBS/BSA, incubated with secondary antibodies for 1 h at 37°C and washed three times for 10 min with PBS. Where appropriate, chromosomes were stained with DAPI (Sigma) for 10 min. After a rinse with PBS, the coverslips were mounted in mowiol (Hoechst, Frankfurt, Germany) containing the anti-fading reagent 1.4-diazabicyclo-(2.2.2)octane (DABCO; Sigma) at 100 mg/ml (Langanger et al., 1983). All antibodies were diluted in PBS/BSA.

Isolated HeLa chromosomes were fixed in 0.8% paraformaldehyde in HEN-buffer (10 mM Hepes, 100 mM NaCl, and 1 mM EDTA, pH 7.4) for 15 min, then sedimented onto coverslips by 5 min centrifugation at 3,000 rpm in a Sorvall HB4 rotor. The coverslips were incubated for 10 min in 0.5 mg/ml NaBH4 in HEN, washed two times with HEN, blocked for 30 min with 10% normal goat serum in HEN and incubated 1 h at room temperature with primary antibodies. After washing with HEN, coverslips were incubated 1 h at room temperature with secondary antibodies, washed two times 5 min and mounted in 50% glycerol, 2% mercaptoethanol in HEN.

All secondary antibodies (rendered species-specific by cross-adsorption) used for the immunofluorescence studies were made in goat and conjugated to AMCA, FITC, and rhodamine (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or Cy3 (Sigma). Controls were performed to ensure that signals from Cy3 labeled structures did not pass into the FITC channel and vice versa. Control coverslips labeled with either nonimmune IgGs or secondary antibodies alone gave only very weak background staining.

Silver Enhanced Immunogold Labeling for EM

Caco-2 cells grown on coverslips were treated for 4 h with 10 μM vinblastine and fixed for 20 min in 3% paraformaldehyde in PHEM-buffer at room temperature, washed three times 5 min in PHEM and permeabilized for 90 s in methanol at -20°C. After a rinse in PBS and two 10 min washes in PBS/BSA, (PBS, containing 0.05% BSA and 0.02% NaN3), the coverslips were incubated overnight at room temperature with rα55 in TBS/ BSA containing 0.2% coldwater fish skin gelatin (FSG; Sigma). After three 10 min washes with PBS/BSA/FSG they were incubated with goat anti-rabbit antibodies coupled with 1 nm colloidal gold (AuroProbe One GAR; Amersham Corp.) diluted in PBS/BSA/FSG for 2 h at 37°C. Postfixation, silver enhancement of gold-particles, further processing and electron microscopy was essentially as described before (Merdes et al., 1991).

Microscopy Techniques

Most images were recorded with a Leica TCS4D confocal microscope. The preparations were sequentially scanned at each Z-position with optimal excitation and filter combinations for UV excitation and blue fluorescence (AMCA or DAPI), and Arg/Crypt laser excitation for FITC (488 nm) and Cy-3 fluorescence (548 nm). The image stacks were stored in crude format on optical memory disks. From optical sections with 0.4 μm vertical pitch, maximal pixel intensity projections were calculated with the help of NIH-Image software. The projections were imported into Adobe Photoshop for pseudocoloring and overlaying. For isolated chromosomes only one optical section was recorded from each field. Then one channel was changed to the DIC-mode to document the appearance of the whole chromosome structure. Whenever appropriate, images were recorded with a conventional multimodal epifluorescence microscope, equipped with a Photometrics cooled CCD camera, controlled by ONCOR software running on Apple OS 7.5. All the images were printed using a Kodak Color Ease color printer.

Results

CLIP-170, Cytoplasmic Dynein, and Dynactin Partially Codistribute during Mitosis

In previous immunolocalization studies we noticed that, in mitotic cells, CLIP-170 was present in dots reminiscent of kinetochores. Their number and staining intensity, however, appeared variable. To find out whether CLIP-170 displayed a dynamic association with kinetochores, we used double immunofluorescence labeling to examine its subcellular distribution relative to centromeres and chromosomes in A431 cells in various stages of mitosis (Fig. 1). Similar results were obtained in HeLa, Cos-7, PtK2, Caco2, and MDCK cells (not shown). In interphase cells,

CLIP-170 was distributed as longitudinal streaks which correspond to the known labeling of MT plus ends (Rickard and Kreis, 1990; Wacker et al., 1992). In prophase cells (Fig. 1, a–d), CLIP-170 staining was found around the centrosomes in a very fine punctate distribution and appeared to be excluded from the nuclear space. It was unclear whether centrosomes themselves were labeled. No MT labeling was seen. In very early prometaphase cells (Fig. 1, e–h), paired CLIP-170–positive dots of varying intensity could be seen on the two sides of almost every centromeric region (Fig. 1 g, inset). During prometaphase progression, the number of CLIP-170–positive centromeres decreased (Fig. 1, i–l). The spindle became more strongly labeled with a fine punctate pattern. In metaphase and anaphase spindles (Fig. 1, m–p), CLIP-170 staining of centromeres could not be detected or was strongly diminished. The fine punctate labeling in the spindle was distributed diffusely throughout the spindle region. CLIP-170 did not appear to accumulate at midbodies in telophase cells (not shown).

The distribution of CLIP-170 next to the centromeric regions (see Fig. 1 g, inset) suggested a recruitment to prometaphase kinetochores. In cells induced to arrest in pseudoprometaphase with vinblastine or nocodazole, (at concentrations sufficient to induce complete depolymerization of MTs), all the centromeric regions stained more strongly for CLIP-170 (Fig. 1, q and r), like has been shown for dynein and dynactin (Echeverri et al., 1996). In such cells, CLIP-170 was found by silver-enhanced immunogold staining to associate with the kinetochore (Fig. 1 s). A more detailed study of the exact localization of CLIP-170 within the kinetochore is being carried out.

The distribution of CLIP-170 at kinetochores was strikingly similar to the distribution of cytoplasmic dynein and dynactin (Echeverri et al., 1996). Therefore, we decided to
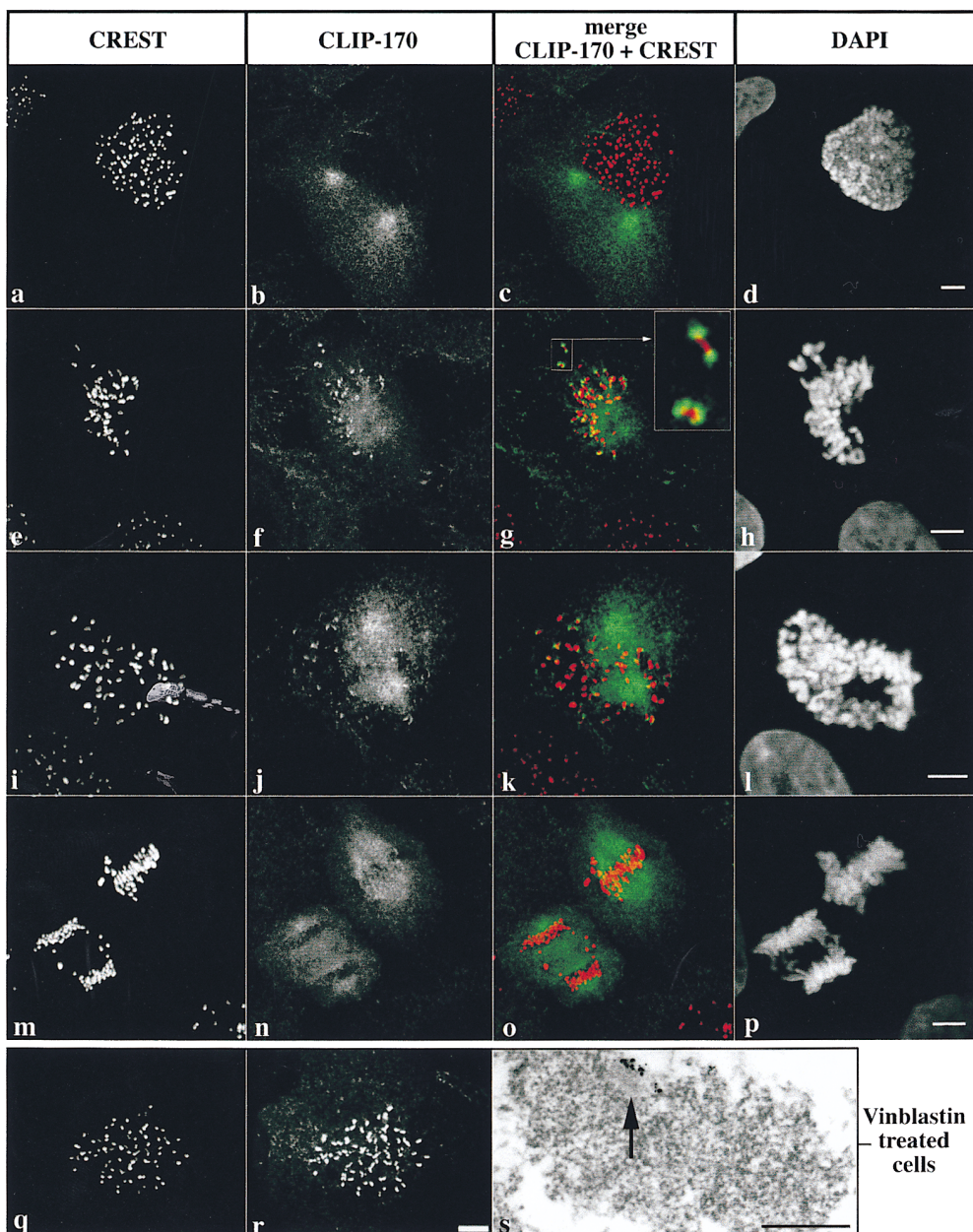

Figure 1. Subcellular distribution of CLIP-170 throughout the mitotic cycle of human A431 cells. (*a*, *e*, *i*, *m*, and *q*) CREST autoimmune staining; (*b*, *f*, *j*, *n*, and *r*) anti–CLIP-170 staining (pAb rα55); (*c*, *g*, *k*, and *o*) superimposition of the *CREST* (*red*) and *CLIP-170* (*green*) signals. (*d*, *h*, *l*, and *p*) DAPI DNA staining. (*a–d*) Prophase cells. (*e–h*) Early prometaphase cells. The inset in *g* shows the relative location of CREST antigens and CLIP-170. (*i–l*) Late prometaphase. (*m–p*) Metaphase cell (*top*) and anaphase cell (*bottom*). (*q* and *r*) Caco2 cell treated with vinblastine. Note the strong CLIP-170 signal at centromeric regions. All the images are maximal projections of optical section stacks. (*s*) thin section of a vinblastine-treated Caco2 cell labeled for CLIP-170 with pAb rα55 and silver-enhanced immunogold staining. Silver particles accumulate at the kinetochore. The cells were fixed in formaldehyde followed by detergent extraction (or cold methanol treatment in *s*). Bars: (*a–r*) 5 µm; (*s*) 0.05 µm.

study the possible codistribution of these proteins in prometaphase cells by triple labeling with antibodies to CREST antigens, CLIP-170, and cytoplasmic dynein or dynactin. CLIP-170 appeared to codistribute with dynactin (Fig. 2, *a* and *b*) and cytoplasmic dynein (Fig. 2, *f* and *g*), and in metaphase cells, the three proteins could not be detected at kinetochores of aligned chromosomes (Fig. 2, *c* and *d*). None of these proteins have been shown to associate stably with each other in cytosol suggesting that they might be recruited independently to kinetochores at the onset of prometaphase. To gain some understanding as to the order of binding we closely inspected, by confocal microscopy, 10 cells labeled for Arp1 and CLIP-170. Of 172 kinetochores scored, nearly all (167) were positive for both CLIP-170 and Arp1. Though staining for both proteins varied in intensity from kinetochore to kinetochore the two appeared to vary in parallel.

CLIP-170 was also detected in the spindle, in the form of a fine, punctate pattern, but did not display the typical localization of dynactin, which is concentrated in a characteristic crescent-like position at the focused minus ends of spindle MTs, colocalizing with dynein and NuMa protein

(Gaglio et al., 1996, Merdes et al., 1996; see color insets Fig. 2).

CLIP-170 Is Present at Kinetochores of Isolated Chromosomes

To verify that the CLIP-170 antibodies were labeling CLIP-170 rather than an immunologically related protein, isolated chromosomes were analyzed by immunofluorescence and immunoblotting. Even after extensive chromosome purification (on percoll gradients, see Gasser and Laemmli, 1987) the kinetochores remained positive for CLIP-170 in a kinetochore-like pattern (Fig. 3, *a–c*). In immunoblots of proteins present in two different purified chromosome fractions, a 170-kD protein was detected by CLIP-170 antibodies in both types of chromosomes (Fig. 3, *d–f*). This suggests that the protein localized to kinetochores by immunofluorescence is indeed CLIP-170. It also appears that only a small fraction of the total CLIP-170 pool is associated with kinetochores of mitotic cells. This is in agreement with previous results showing that CLIP-170 is largely cytosolic (Rickard and Kreis, 1991). The 170-kD
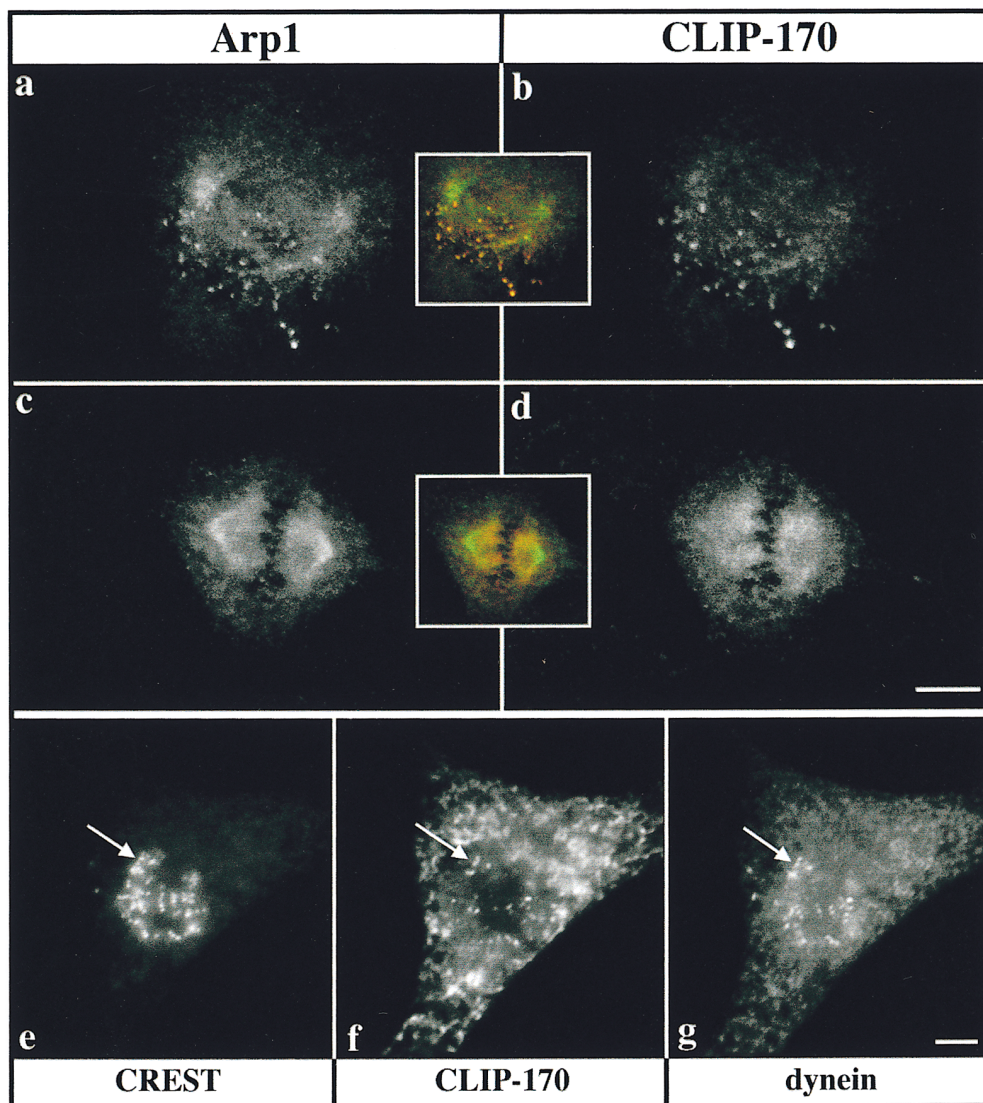

Figure 2. CLIP-170, cytoplasmic dynein and dynactin components transiently colocalize at kinetochores of nonaligned chromosomes. (*a* and *c*) Anti-dynactin (Arp1) staining; (*b*, *d*, and *f*) Anti–CLIP-170 staining (monoclonal anti–CLIP-170, 4D3 in *b* and *d*, rabbit anti–CLIP-170 [rα55] in *f*); (*g*) anti-cytoplasmic dynein intermediate chain staining. (*e*) CREST autoimmune staining. (*a–d*) Cells were fixed in paraformaldehyde and permeabilized in cold methanol. *a* and *b* show the colocalization of Arp1 and CLIP-170 at kinetochores of a prometaphase cell. Staining is no longer seen at aligned chromosomes in a metaphase cell (*c* and *d*). The color insets show the overlay of CLIP-170 (*red*) and Arp1 signals (*green*). Note the relative absence of CLIP-170 concentration in the polar regions. (*e–g*) cells were fixed in −20°C methanol. Arrows show the same kinetochores labeled for cytoplasmic dynein and CLIP-170 in a prometaphase cell. Similar results were obtained with a monoclonal against p150[GLUED] (not shown). *a–d* are complete maximal projections of optical section stacks. *e–g* were taken using a cooled CCD camera. Bar, 5 μm.

band is more weakly detected in percoll purified chromosomes (lane 4) by both CLIP-170 antibodies, but a band of ~40 kD was newly detected by 4D3. Thus, it appears that CLIP-170 was partly proteolyzed in percoll purified chromosomes and that the 40-kD band most likely corresponds to a proteolytic fragment of CLIP-170. Rα55, raised against and purified on a CLIP-170 fusion protein comprising amino acids 211–854 (clone 55, see Pierre et al., 1992), did not recognize this polypeptide. 4D3 being directed against an epitope located in the COOH-terminal part of the coiled coil region, we infer that the 40-kD fragment seen in lane 4, but absent in lane 3, corresponds to a COOH-terminal fragment of CLIP-170. The fact that it remained associated with the kinetochores suggests that this domain could be responsible for its targeting.

Analysis of CLIP-170 Targeting

To determine which of the domains of CLIP-170 might be responsible for kinetochore targeting, different deletion constructs, encoding myc-tagged proteins were introduced into COS-7 cells which were transfected by standard methods. Proteins lacking the MT-binding domain (ΔN), the MT-binding domain and much of the predicted coiled-coil rod (ΔNΔR), the putative COOH-terminal cargo binding domain (ΔC), and the MT-binding domain and the COOH-terminal domain (ΔNΔC) were tested for their
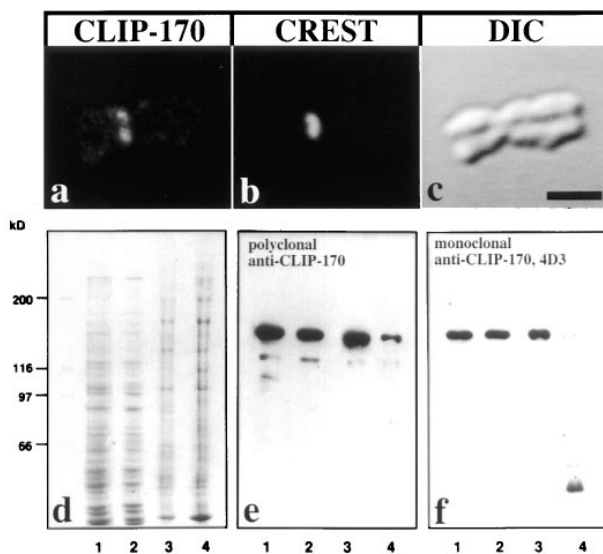

Figure 3. (*a–b*) CLIP-170 staining of isolated HeLa chromosomes. Chromosomes were isolated from colcemid-treated HeLa cells, then labeled with anti–CLIP-170 (pAb rα55) (*a*) and CREST auto-immune serum (*b*) and imaged by confocal microscopy (*a and b*) and differential interference contrast microscopy (*c*). In *a and b*, a single optical section is shown. (*d–f*) Coomassie blue stained gel (*d*) and Western blots (*e* and *f*), of cell fractions in the course of chromosome purification. Lane *1*, total cell extract; lane *2*, cytosol; lane *3*, chromosomes purified on a glycerol gradient; lane *4*, chromosomes purified on a Percoll gradient. The amounts of the samples used were adjusted to yield comparative immune signals and do not reflect the quantitative distribution of CLIP-170 in the various fractions. (*e*) Labeling with anti–CLIP-170 serum rα55; (*f*) labeling with anti–CLIP-170 mAb 4D3. The lower band in *f*, lane *4* is a COOH-terminal proteolytic fragment of CLIP-170, that is not recognized by rα55. Bar, 2 μm.

ability to bind kinetochores. Previous work aimed at characterizing functional domains of CLIP-170 in interphase cells indicated that the myc tag did not alter the behavior of any of these molecules (Pierre et al., 1994). To detect the kinetochore-bound CLIP-170, excess cytosolic protein was removed by a prelysis step. By observing Myc-positive kinetochores in early prometaphases or those of lagging chromosomes in cells in prometaphase, it was found that full length CLIP-170 (Fig. 4, *a–c*), ΔN (Fig. 4, *d–f*), and ΔNΔR (Fig. 4, *g–i*) all bind kinetochores. Absence of binding for ΔC and ΔNΔC was inferred from the observation that in parallel experiments, no mitotic cells displaying Myc-labeled kinetochores were found. To obtain stronger evidence for the absence of kinetochore-binding of these forms these experiments were repeated using A431. The deletion constructs were introduced into synchronized cells by microinjection. Before fixation, these cells were treated with nocodazole (see Material and Methods) in order to have some of the injected cells in mitosis by the time of fixation and to obtain kinetochores displaying strong and uniform signals for kinetochore-binding forms of CLIP-170 after cell lysis. The overexpressing cells were relocated within the grid pattern on the CELLocate coverslips on the basis of their coordinates and the remaining cytoplasmic immunofluorescent signal. Analysis by confocal microscopy confirmed kinetochores binding of wt-CLIP-170, ΔN and ΔNΔR (not shown). ΔC and ΔNΔC (Fig. 4, *j–l* and *m–o*) appeared not to bind at the kinetochore. Other proteins have been found to associate artifactually with the centromere in cells arrested in pseudoprometaphase by the use of MT depolymerizing drugs (Compton et al., 1991). It is therefore very important not to use the procedure outlined for A431 cells as a primary method for mapping kinetochore-targeting domains of proteins.

The above results suggest that all or part of the COOH-terminal 152 amino acids of CLIP-170 constitute the kinetochore-binding domain.

The overexpression of dynamitin, the dynactin subunit thought to link the dynein and cargo binding domains (reviewed in Schroer, 1996, Vallee and Sheetz, 1996) inactivates dynactin function (Echeverri et al., 1996). The targeting of at least one kinetochore-associated protein, cytoplasmic dynein, is inhibited by dynamitin overexpression, although it does not cause nonspecific disruption of kinetochore structure, as the association of CENP-E with kinetochores is not affected (Echeverri et al., 1996). Therefore, we determined whether or not CLIP-170 binding to kinetochores was altered in cells overexpressing dynamitin. COS-7 cells were transiently transfected with a chicken dynamitin cDNA, then treated with nocodazole before fixation (see Materials and Methods). As previously reported (Echeverri et al., 1996), dynamitin overexpression caused a reduction in the amount of dynactin and dynein at kinetochores (not shown). In a large majority of the cells overexpressing dynamitin (Fig. 5, *a–c*) CLIP-170 was also strongly reduced at kinetochores, often to levels below the sensitivity of our detection method, whereas in transfected cells that were not overexpressing dynamitin (right cell in Fig. 5, *d–f*) CLIP-170 staining was unaffected. In a small minority of dynamitin overexpressing cells (left cell in Fig. 5, *d–f*) some CLIP-170 and Arp1 labeling of kinetochores
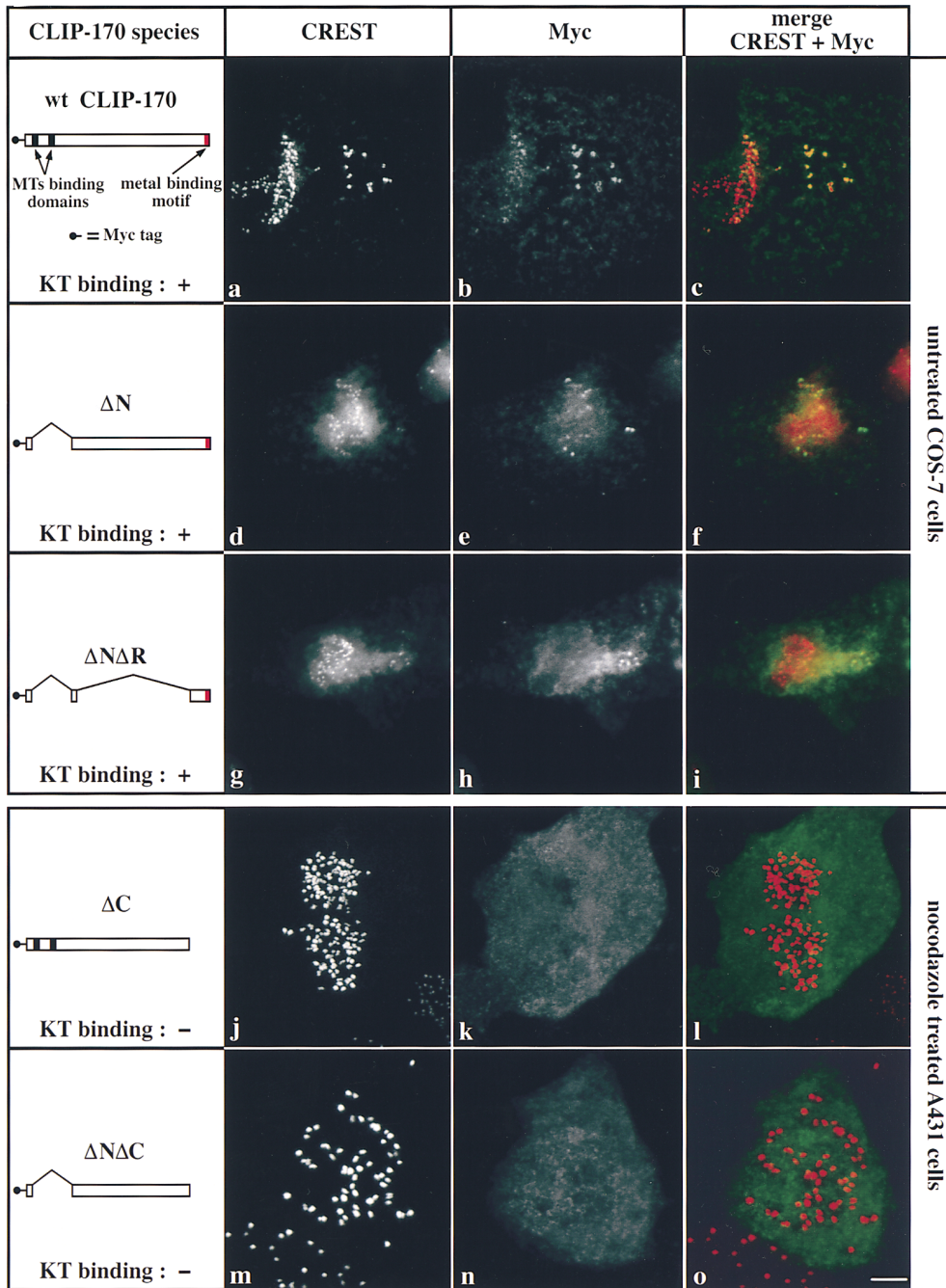
Figure 4. CLIP-170 species that possess the COOH-terminal domain can bind kinetochores. a–i show COS-7 cells transiently transfected by standard techniques. j–o show A431 cells seeded on CELLocate coverslips, synchronized and intranuclearly microinjected with plasmids, and then treated with nocodazole before fixation and processing for immunofluorescence. The fixation in 3% paraformaldehyde was preceded by extraction in Triton X-100, in order to render kinetochore-bound CLIP-170 detectable. The cells were double labeled with CREST autoimmune serum (a, d, g, j, and m) and anti-myc (b, e, h, k, and n). c, f, i, l, and o are superimpositions of CREST (red) and myc (green) signals. The A431 cells were relocated on the coverslip. Myc staining is seen at kinetochores of nonaligned chromosomes in cells transiently overexpressing wt-CLIP-170 (a–c), ΔN (d–f), and ΔNΔR (g–i). CLIP-170 species lacking the COOH-terminal domain (ΔC [j–l] and ΔNΔC [m–o]) do not appear to bind kinetochores. The panels at the left of the figure are diagrams representing the constructs used. All the panels, except d–i are complete maximal projections of x/y optical section stacks. d–i were taken using a cooled CCD camera. Bar, 5 μm.

was still detected, similar to what was reported previously for the dynactin subunit Arp1 (Echeverri et al., 1996).

Phenotypic Effects of Overexpression of Dynamitin or CLIP-170 Mutants Capable of Accumulating at the Kinetochore

As a first step in analyzing CLIP-170 function during mitosis in vivo, we transiently transfected COS-7 cells with CLIP-170 species that had the capacity to bind kinetochores (wt-CLIP-170, ΔN, ΔNΔR). As controls, the cells were transfected with dynamitin or the CLIP-170 mutant ΔNΔC which cannot bind to either MTs or kinetochores. Cells stained for MTs, chromosomes, and the overex-

pressed protein were used to determine the mitotic index and the mitotic phase index of expressing and nonexpressing cells in the transfected population (Fig. 6). For each construct, at least 3,000 cells were examined, except for ΔNΔC, where >50,000 cells were scored. All the kinetochore-binding forms of CLIP-170 yielded increased numbers of cells in mitosis. Roughly 7% (∼6% for dynamitin) of the cells overexpressing these species were in M-phase, as compared with ∼3.5% for the nonexpressing cells. In contrast, the mitotic index of cells overexpressing ΔNΔC was <0.2%, suggesting a block of entry into mitosis (see Fig. 6 A).

To determine whether a specific stage of mitosis was affected by the overexpression of these CLIP-170 forms, we
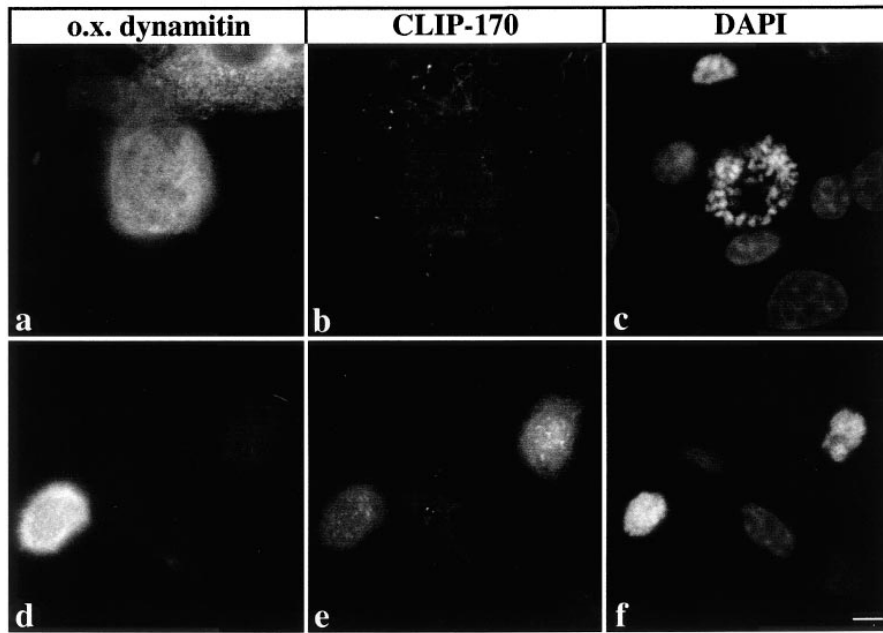
| o.x. dynamitin | CLIP-170 | DAPI |

Figure 5. Effect of overexpressing dynamitin on anti–CLIP-170 staining of kinetochores. COS-7 cells transfected with chicken dynamitin were nocodazole-treated and double stained with anti-dynamitin (*a* and *d*), monoclonal anti–CLIP-170 (*b* and *e*), and DAPI for DNA staining (*c* and *f*). (*a–c*) A mitotic cell, representative of the majority of the cells overexpressing dynamitin. No CLIP-170 signal is visible at kinetochores. (*d–f*) mitotic cell to the right, not overexpressing dynamitin: normal CLIP-170 staining at the kinetochores. Mitotic cell to the left, overexpressing dynamitin: diminished CLIP-170 at the kinetochores, a phenotype seen in a small subpopulation of cells. The cells were fixed in formaldehyde followed by detergent extraction. The images were taken using a cooled CCD camera. Bar, 10 μm.

scored the mitotic phase indexes of ~200 mitotic expressing and nonexpressing cells in each transfected population (~100 mitotic cells were scored for ΔNΔC). An increased number of prometaphase figures were detected in cells expressing kinetochore-binding CLIP-170 forms: ~74% (see Fig. 6 *B*) showed a pseudoprometaphase chromosome configuration, compared with ~35% of prometaphase configurations in control mitotic cells. This suggested that progression through prometaphase was delayed.

The transient transfection approach used in this study often exhibits cell to cell variability. Under the conditions used in our study, only the cells containing readily detectable, evenly distributed CLIP-170 reactivity, indicative of medium to high levels of expression, were scored. Except when indicated, all the observations made during the functional characterization of the phenotype described below were seen in the vast majority of the analyzed cells. We infer that even a moderate overexpression of the CLIP-forms used here induces the observed effects.

Cells overexpressing kinetochore binding forms of CLIP-170 appeared to be able to assemble spindles with distinct poles and astral MTs (shown for ΔNΔR, a mutant
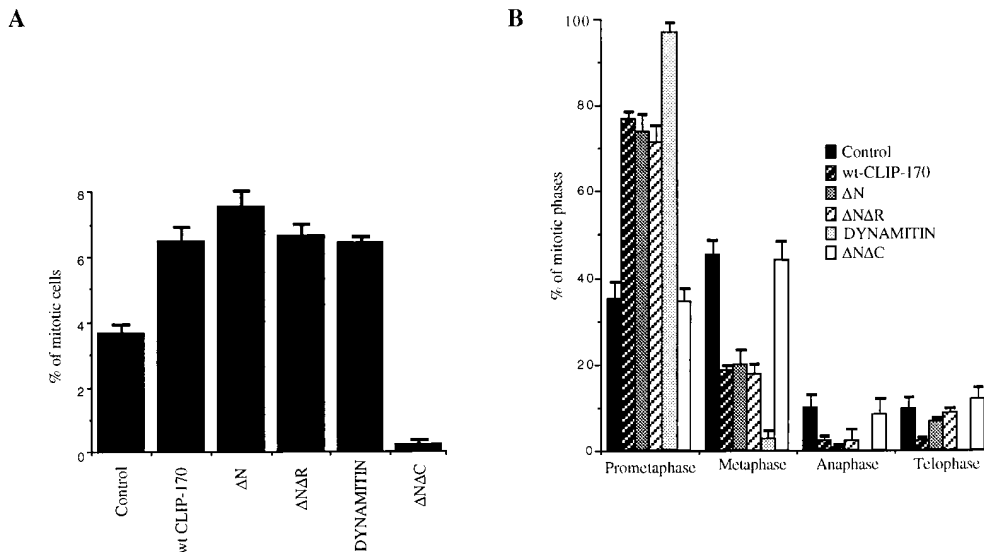

Figure 6. Effect of overexpression of CLIP-170 deletion mutants on mitotic index (*A*) and mitotic progression (*B*). COS-7 cells were transfected with constructs of CLIP-170 or dynamitin. (*A*) The mitotic indexes of cells overexpressing dynamitin and the CLIP-170 species addressed to the kinetochore were significantly different from the control value in transfected, nonoverexpressing cells, whereas that for ΔNΔC–CLIP-170 indicates a block of entry into M-phase. (*B*) Mitotic phase indexes of nonoverexpressing cells in the transfected population (control, *black bars*), and of cells overexpressing dynamitin or various CLIP-170 deletion mutants. Cultures stained with anti–CLIP-170, anti-tubulin, and DAPI were scored for mitotic phase on the basis of chromosome configurations and spindle morphology. All values are means from three independent experiments. At least 3,000 transfected cells (mitotic and nonmitotic) were counted for each construct. For ΔNΔC, more than 50,000 cells were counted because of the very low mitotic index (O.19%). Values for the dynamitin construct were comparable to those of a previous study (Echeverri et al., 1996). Those for wt-CLIP-170, ΔN and ΔNΔR were found to be significantly different from those for ΔNΔC and for transfected nonoverexpressing control cells ($P < 0.05$, according to Student's *t* test). The latter were not significantly different from the values for untransfected cultures (not shown).

that contained the COOH-terminal kinetochore binding domain but little else in Fig. 7, *b* and *c* and Fig. 8). As shown in Fig. 7 for representative cells, a large majority (86.3% ± 5.5% SD or 63% of the mitotic cells) of the overexpressing pseudoprometaphase cells displayed spindle shapes very similar to that found in about half (40% ± 4.2% SD or 14% of the mitotic cells) of the nonexpressing prometaphase cells in the same population. In such cells, spindle pole separation was limited, the spindles appeared small and compact, additional pole spurs were observed and the spindle axis appeared randomly oriented. The elevated frequency of this spindle morphology in nonexpressing prometaphase COS-7 cells indicates that it does not represent an abnormal state, but is instead characteristic for cells accomplishing the early stages of spindle assembly in this cell line. In the transfected cell population, ~19% of the mitotic cells had constructed metaphase-like spindles, with most of the chromosomes aligned and hypercondensed (see Fig. 8, *i* and *j*). Anaphase and telophase configurations were also found, but more rarely (Fig. 6 *B*).

In cells overexpressing wt-CLIP-170, large CLIP-170 containing aggregates adjacent to the spindle were also frequently found; these did not stain with tubulin antibodies (not shown). The mitotic phase index of cells overexpressing CLIP-170 ΔNΔC was identical to that of control cells (see Fig. 6 *B*), showing that there was no additional delay in prometaphase. In cultures transiently overexpressing dynamitin (included as a control) we observed similar phenotypic effects to those originally reported by Echeverri et al. (1996; see also Fig. 6 *B*), with nearly all the mitotic cells in a pseudoprometaphase configuration.

Since the deletion mutant ΔNΔR accumulates at kinetochores (see Fig. 4, *g–i*), it might compete with and displace endogenous CLIP-170 from kinetochore-binding sites, leading to a dominant negative inhibition of processes in which CLIP-170 is involved. To test this possibility, we localized endogenous CLIP-170 in cells overexpressing ΔNΔR using two antibodies that allow endogenous and exogenous CLIP-170 species to be distinguished (see Materials and Methods). In the vast majority of the cells overexpressing ΔNΔR, the amount of endogenous CLIP-170 at early prometaphase or lagging kinetochores (Fig. 8, *a–c*) appeared reduced, very often to levels below the sensitivity of our detection method, whereas in nonexpressing cells in the same transiently transfected population, kinetochore staining at similar chromosomes was strong (Fig. 8, *d–f*). We infer from this result that ΔNΔR displaces endogenous CLIP-170 from prometaphase kinetochores, and that this is the case in nearly all the cells displaying medium to high expression levels.

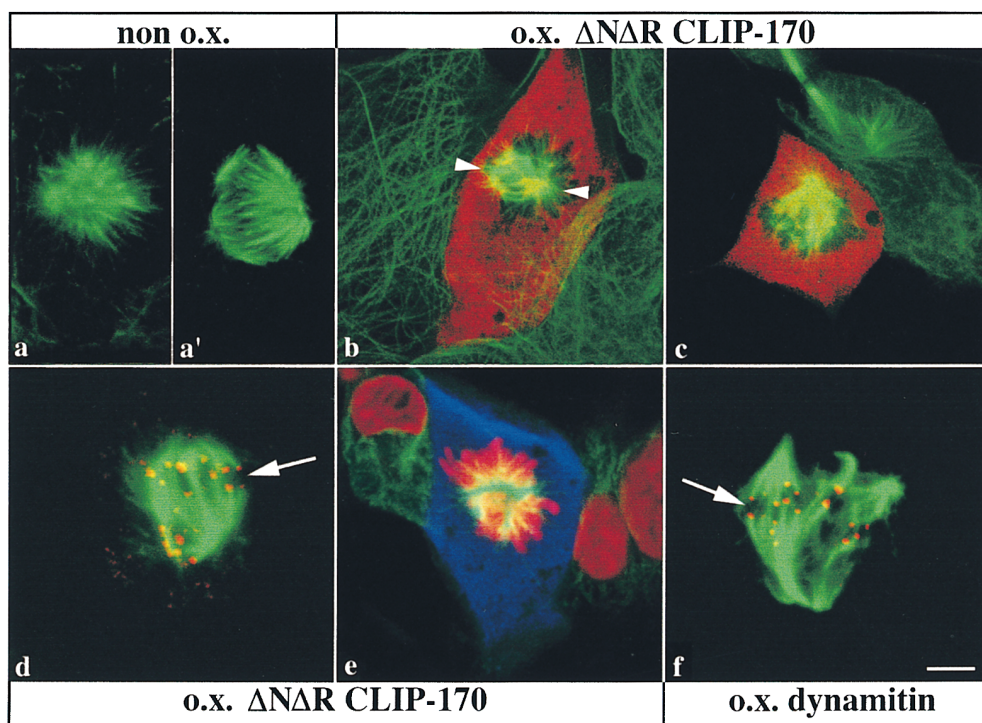It also seemed possible that the presence of ΔNΔR at the kinetochores of overexpressing cells might affect the

Figure 7. Analysis of the phenotypic effects of ΔNΔR and dynamitin overexpression on spindle morphology. (*a–c*) ΔNΔR transfected COS-7 cells were stained for tubulin (*green*) and overexpressed CLIP-170 (pAb rα55, *red*); (*a* and *a'*) show the spindle of two nonexpressing early prometaphase cells in the transfected population. (*b* and *c*) show the spindles of two ΔNΔR overexpressing cells that were scored as being in pseudoprometaphase. The images show complete maximal projections of optical section stacks. The ΔNΔR staining is provided as a single optical section to allow for the localization of chromosomes. The cells were fixed in 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.05% Triton X-100. (*d–f*) Analysis of the phenotypic effects of ΔNΔR and dynamitin overexpression on chromosome/spindle interactions. COS-7 cells were transfected with ΔNΔR (*d* and *e*) or dynamitin (*f*) then labeled with antibodies for tubulin, the CREST antigen and the transfected protein (*d* and *f*), or with antibodies to tubulin, the transfected protein and DAPI for the chromosomes (*e*). *d* and *f* are superimpositions of selected single optical x/y sections of the CREST (*red*) and tubulin signals (*green*). Labeling for the transfected proteins is not shown but was used to identify transfected cells. The kinetochores are apparently correctly attached to kinetochore fibers despite the overexpression of ΔNΔR or dynamitin. *e* represents a superimposition of three single optical sections showing tubulin (*green*), ΔNΔR (*blue*), and DNA (*red*) signals. The labeling of the chromosomes shows that their arms are correctly oriented outward. The cells in *e* were fixed in 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.05% Triton X-100. The cells in *d* and *f* were fixed with the same mixture, except that 0.01% glutaraldehyde was used. Bar: (*a–d*) 5 μm; (*e* and *f*), 2.5 μm.
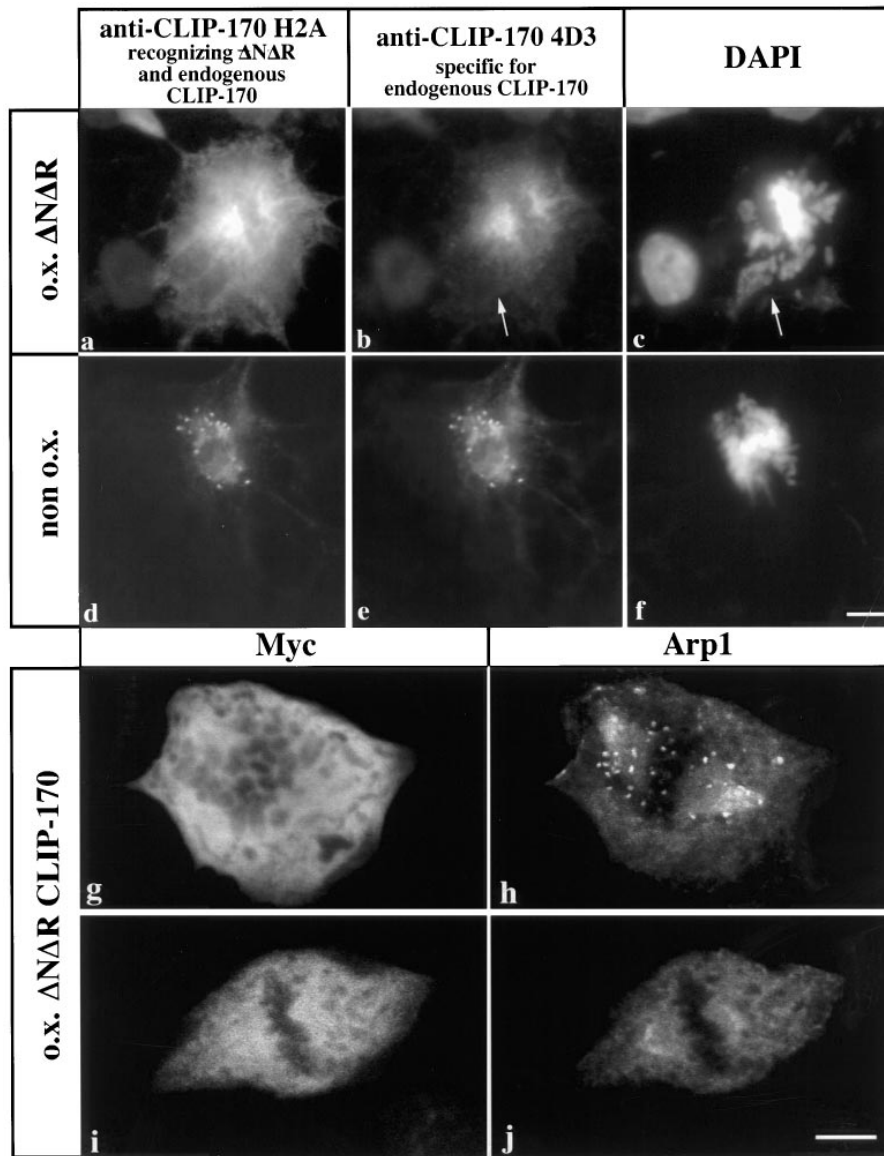
Figure 8. Overexpression of ΔNΔR displaces endogenous CLIP-170 from kinetochores, but has no effect on the transient kinetochore localization of dynactin (*Arp1*) and ΔNΔR. COS-7 cells were transfected with ΔNΔR and fixed in 3% paraformaldehyde, followed by permeabilization with detergent (*a–f* and *k–m*) or cold methanol (*g–j*). Cells were labeled for the transfected ΔNΔR with the anti–CLIP-170 pAb H2A (*a* and *d*) or with monoclonal anti–c-myc (*g, i,* and *l*), for endogenous CLIP-170 with a monoclonal, 4D3 (*b* and *e*), which does not recognize the transfected protein, for chromosomes with DAPI (*c, f,* and *m*) and for Arp1 (*h* and *j*). Overexpression of ΔNΔR leads to diminished staining of nonaligned kinetochores for the endogenous protein with 4D3 (*a–c*), whereas in nonexpressing cells, 4D3 strongly labels such kinetochores (*d–f*). (*g–j*) Arp1 labeling of cells expressing ΔNΔR shows that the targeting of this protein to prometaphase kinetochores (*g* and *h*) as well as its disappearance from aligned chromosomes (*i* and *j*) is unaffected by overexpression of ΔNΔR. Panels (*a–f*) are images taken using a cooled CCD camera. *g* and *i* are single optical sections of the ΔNΔR signal and *h* and *j* are maximal projections of the Arp1 signal. Bars, 5 μm.

binding of dynein and/or dynactin at kinetochores of early prometaphase chromosomes. Its presence in the cell may also affect the accumulation of these proteins at the spindle poles. Finally, it might also interfere with the loss of dynein, dynactin or its own staining from kinetochores of aligned chromosomes. Dynein (not shown) and dynactin (shown for Arp1, Fig. 8, *g–j*) accumulated normally at the polar region and were detected on kinetochores of unaligned chromosomes (Fig. 8, *g* and *h*) but were strongly reduced or no longer detectable on aligned chromosomes (Fig. 8, *i* and *j*), exactly as in nonoverexpressing cells. In ΔNΔR overexpressing cells prelysed with Triton X-100, we observed that this CLIP-170 form associated with kinetochores of unaligned chromosomes and was no longer detectable on aligned chromosomes (Fig. 4, *g–i*), thus itself displaying the typical dynamic kinetochore association characteristic for endogenous CLIP-170 (see Fig. 8, *d–f*). The same was found for wt-CLIP-170 (Fig. 4, *a–c*) and ΔN (see Fig. 4, *d–f*).

Thus, the phenotypic effect induced by this minimal kinetochore-binding domain of CLIP-170 is most likely not caused by an effect on the localization of dynein/dynactin to kinetochores or to poles, or by an inhibition of its dynamic association with kinetochores, but rather by the replacement of endogenous CLIP-170 by ΔNΔR at the kinetochore.

Because CLIP-170 might participate in the end-on attachment of kinetochore MTs to the outer plate of the kinetochore, the presence of a CLIP-170 mutant that was incapable of binding to tubulin might be expected to affect kinetochore fiber formation. To address this question, we analyzed the interaction of kinetochores with MTs in cells overexpressing the CLIP-170 deletion construct ΔNΔR. Overexpressing cells were triple stained for MTs, centromeres and CLIP-170 ΔNΔR and imaged by high resolution confocal microscopy. Kinetochore fibers connecting centromeres and poles were clearly evident (Fig. 7 *d*), as they were in cells overexpressing other kinetochore-binding forms of CLIP-170 (wt-CLIP-170 and ΔN; not shown). The chromosomes appeared to be fully integrated within the spindles in a bent configuration with arms pointing away from the spindle midzone, again suggesting that the

chromosomes were connected to the poles by bundles of kinetochore MTs (Fig. 7 *e*).

Overexpression of dynamitin in COS-7 cells caused similar spindle aberrations to those reported previously (Echeverri et al., 1996; Gaglio et al., 1996). We noted that the effects of dynamitin overexpression on MT organization in mitotic spindles were much more dramatic than those caused by overexpressing CLIP-170 mutants, presumably due to the additional distortion of polar regions. In this original study, centromeres (visualized by CREST staining) were seen to colocalize at random with spindle MTs and it was not clear whether the kinetochores were associated with the tips of bundles of kinetochore MTs. In triple staining experiments (for centromeres, MTs and dynamitin) most of the CREST stained centromeres appeared to be tethered at the tips of two MT fibers (Fig. 7 *f*).

Of the possible interpretations of our combined results, we favor the one indicating that the overexpressing cells need more time to accomplish the early stages of spindle assembly, but ultimately assemble functional spindles. This phenotype is therefore novel.

Discussion

Subcellular Codistribution of CLIP-170, Cytoplasmic Dynein, and Dynactin at Prometaphase Kinetochores during Mitosis

The MT binding protein CLIP-170 was first identified as a cytoplasmic linker protein docking endocytic vesicles to MTs (Rickard and Kreis, 1996). We show here that CLIP-170 is also localized at kinetochores of prometaphase chromosomes during mitosis but is lost by the time chromosomes align at the metaphase plate. This pattern of recruitment and loss is very similar to that previously reported for dynein and dynactin and in our hands the three proteins showed nearly identical behavior in the course of chromosome alignment. This dynamic codistribution of CLIP-170 with cytoplasmic dynein and dynactin at prometaphase kinetochores offers the first evidence that the three proteins colocalize within cells on motile organelles. The association of the three proteins may have been detected more easily on prometaphase kinetochores than on endocytotic vesicles, because kinetochore structures are larger and longer-lived (Rieder and Salmon, 1994) than endocytotic vesicles interacting with MTs. CLIP-170 does not clearly colocalize with dynein/dynactin in the characteristic crescent-like position at the focused minus ends of spindle MTs. In addition, its immunodepletion from mitotic extracts prepared from synchronized HeLa cells had no observable effect on either the organization of microtubules into mitotic asters or the efficiency with which Eg5, NuMA, dynactin, and cytoplasmic dynein associated with the MTs (Gaglio, T., and D.A. Compton, personal communication). These observations suggest that CLIP-170 is not required for the centrosome-independent aspects of microtubule minus end organization at mitotic spindle poles.

Our results strongly suggest that CLIP-170 has a function at the kinetochore during the establishment of metaphase chromosome alignment, and in particular during an early step. The function of CLIP-170 remains unknown, but it appears as a reasonable candidate for being involved in the capture of the first MTs by the kinetochore. This process entails a tangential interaction, and not necessarily binding at the plus end, as is the case with the capture of most kinetochore MTs (Merdes and De Mey, 1990; Rieder and Alexander, 1990). In living cells, the capture event is followed without delay by a poleward pulling on the kinetochore, indicating that capture and movement may be linked (Hayden et al., 1990). Our findings also suggest that either dynactin or dynein might be the receptor for CLIP-170 at the kinetochore. This would assure that CLIP-170 would be in a position to interact intimately with components of the motor complex in order to participate in the initiation of movement. Since CLIP-170 appears to stay associated with the dynein/dynactin motor complex during the initial poleward movement, CLIP-170 might also serve to govern dynein activity. It is important to remind that the role of dynein/dynactin in kinetochore motility is not yet clear. Unpublished results from our group indicate that, if this motor indeed plays a role, it is limited to the first phase of poleward kinetochore movement during prometaphase chromosome movements (Dujardin, D., and J.R. De Mey, manuscript in preparation). If CLIP-170 does, in fact, mediate kinetochore docking onto polar MTs and/or govern dynein activity, then capture or initial poleward movement might be altered in cells overexpressing CLIP-170 mutants. We are now analyzing in detail the first phases of MT/kinetochore interaction and of kinetochore motility in ΔNΔR overexpressing cells.

Functional Domains of CLIP-170 and Kinetochore Targeting

The acidic COOH-terminal domain of CLIP-170 has been shown previously to be required for the targeting of CLIP-170 to cytoplasmic structures that interact with peripheral MT plus-ends (Pierre et al., 1994). These results, together with those presented here, provide evidence that the COOH-terminal domain may be a general cargo-binding domain. It would appear that CLIP-170 acts as a MT-cargo linker for both membranous and nonmembranous organelles, serving different cargoes during different stages of the cell cycle. The COOH-terminal domain contains a consensus metal-binding motif, also present in the yeast protein Bik1p (Berlin et al., 1990) and retroviral *gag*-encoded nucleocapsid proteins (Pierre et al., 1992), that could be responsible for the interaction of CLIP-170 with other proteins. Further work will be aimed at determining whether this motif plays a role in the targeting of CLIP-170 to the kinetochore via its interaction with kinetochore components, or alternatively, in the interaction with proteins regulating CLIP-170 function.

Dynactin is believed to mediate the association of cytoplasmic dynein with kinetochores and other organelles (Echeverri et al., 1996). In our experiments, the overexpression of dynamitin results in a reduction of CLIP-170 on prometaphase kinetochores that also have reduced amounts of dynein and dynactin. This suggests that dynactin is involved, either directly or indirectly, in the association of CLIP-170 with kinetochores. Alternatively, excess dynamitin might titrate out a structural or regulatory fac-

tor aside from dynactin or cytoplasmic dynein that is critical for the binding of CLIP-170 to kinetochores. Finally, it is possible that excess dynamitin binds to the kinetochore and masks the CLIP-170–binding site.

Why dynein, dynactin and CLIP-170 staining is ultimately lost from aligned chromosomes is not yet clear. It could reflect epitope masking or a change in antibody accessibility, but this seems unlikely since the same result is obtained for three distinct proteins, using a variety of antibodies and staining conditions, in independent studies performed in different labs. Echeverri et al. (1996) have proposed that the release of these proteins from kinetochores depends on the behavior of individual chromosomes with respect to their interactions with spindle MTs.

Mitotic Phenotype Caused by CLIP-170 Species Recruited at Kinetochores

A role for CLIP-170 in mitosis is supported by our findings that overexpression of species that have the capacity to bind kinetochores but not MTs causes cells to become retarded in prometaphase. The increase in mitotic index was similar to that seen when dynamitin is overexpressed in cells, which is known to disrupt dynein association with the kinetochore, but also to disrupt the dynein/dynactin/NuMa mediated focusing of MT minus ends at the poles. However, our results indicate that these CLIP-170 mutants displace endogenous CLIP-170 from the kinetochore, but not dynein or dynactin from either kinetochore or polar region. It would appear that the MT-binding activity of CLIP-170 is required for normal mitotic progression. Yet we cannot rule out completely that the accumulation of mutated CLIP-170 species at the kinetochores causes disruption of the association of other proteins leading to impaired kinetochore structure and or function. It is also unknown whether and how CLIP-170 contributes to chromosome motility. A hint is provided by our observation that the majority of the overexpressing cells retarded in prometaphase have a spindle morphology corresponding to that of many normal early prometaphase COS-7 cells. This indicates that an early event, possibly the initial, tangential capturing of polar MTs, is affected. However, this does not seem to arrest the cells. This is supported by the observations that (a) the spindles ultimately formed are not disrupted; (b) poles and kinetochore fibers that look normal at the light microscopic level are formed; (c) dynein/dynactin motor complexes accumulate normally in the polar regions; (d) they also appear to be released from aligned chromosomes as usual, and; (e) metaphase spindles formed also seem to be able to progress into anaphase, a process known to be very sensitive to kinetochore malfunction. That the CLIP-170 COOH-terminal domain by itself (i.e., the ΔNΔR mutant) is released from aligned chromosomes further suggests that the elements that regulate the association of CLIP-170 with kinetochores are still operational and situated in the COOH-terminal domain. This novel phenotype holds promise for a better understanding of the role of both CLIP-170 and of dynein/dynactin in the initial phases of poleward chromosome movement.

It is also interesting that Bik1p is the only protein of the Saccharomyces cerevisiae genome presenting homologies to CLIP-170 in both the NH$_2$- and COOH-terminal domains, and may therefore represent the functional CLIP-170 homologue in yeast. Bik1p is required for karyogamy and localizes to spindle MTs, suggesting a role in mitosis (Berlin et al., 1990).

The MT-binding domain of CLIP-170 is thought to be involved in capturing MTs, so it came as a surprise that kinetochore fibers were still formed in cells overexpressing ΔNΔR. Moreover, kinetochore fibers are also formed in dynamitin overexpressing cells whose chromosomes have very reduced levels of both dynein/dynactin and CLIP-170 at the kinetochore. Although these kinetochore fibers looked normal at the light microscopic level, we cannot exclude that they contain more subtle defects. For example, kinetochore fibers have also been seen in cells microinjected before late G2 with anti-centromere autoantibodies, although the internal structure of the kinetochores had been altered and the kinetochores did not move. (Bernat et al., 1991). Therefore, it will be necessary to do serial section EM on the cells retarded in pseudopremetaphase described here, in order to asses the quality of the chromosome/MT connection. Nevertheless, it would appear that neither the dynein/dynactin complex nor CLIP-170 are required for the assembly of at least rudimentary kinetochore fibers, an event that involves an insertion of MT plus ends into the outer kinetochore plate and occurs at a stage of kinetochore motility subsequent to the initial poleward movement (McEwen et al., 1997; Rieder and Salmon, 1994). The assembly of mature kinetochore fibers may therefore proceed via a secondary mechanism that is independent of the dynein motor complex, and uses other protein complexes. Consistent with this, the initial capture/movement event and the ultimate formation and maturation of kinetochore fibers appear to be distinct processes (McEwen et al., 1997).

At first, we were surprised that overexpression of full-length CLIP-170 caused a mitotic phenotype similar to that seen with mutants that lacked the MT-binding NH$_2$-terminal domain. However, we found that the overexpressed protein did not strongly associate with the spindle MTs, but instead frequently accumulated in aggregates which accumulated in the cytoplasm adjacent to the spindle and did not stain with tubulin antibodies (not shown). In interphase cells, overexpressed full-length CLIP-170 accumulates along MTs that have become rearranged into thick bundles, or in aggregates that also contain tubulin (Pierre et al., 1994). Therefore, it would appear that, upon entry into mitosis, overexpressed full-length CLIP-170 is released from the depolymerizing interphase MTs and becomes sequestered in a form that is diminished in its capacity to bind the dynamic spindle MTs. But how could we explain that this may also be the case for the kinetochore-bound fraction? Binding of CLIP-170 to MTs is inhibited by hyperphosphorylation (Rickard and Kreis, 1991). One of several possible mechanisms therefore is that wt-CLIP-170 overexpression titrates out a factor active in mitotic cells (e.g., a phosphatase or a kinase inhibitor), and that this may ultimately be leading to its hyperphosphorylation. This would inhibit the MT-binding capacity of the kinetochore-bound fraction, leading to the same effects as overexpression of CLIP-170 species that lack the MT-binding region altogether. If this mechanism were to be

true, this phenotype would provide strong support for the prometaphase phenotype described here being kinetochore based.

So far, CLIP-170 has not been found to bind any cytosolic proteins other than tubulin. The dynamic codistribution of CLIP-170 with cytoplasmic dynein and dynactin at prometaphase kinetochores, and its dependence on dynactin or dynein for kinetochore binding suggest that it may display carefully controlled interactions with the dynein/dynactin complex. Much remains to be learned about how the initial static link between cargo and MT is converted into a state that supports movement. Our data clearly indicate that a complete understanding of the mechanism by which dynein-mediated motility is initiated in vivo requires determination of the relative contributions of dynactin and CLIP-170 to the initial binding of cargo to MT, as well as further study of how the activities of the motor complex and CLIP-170 are coordinated. The kinetochore will provide a useful model for this problem. A complete understanding of how CLIP-170 contributes to these processes should thus provide new insights into the mechanism of mitosis and also of dynein-mediated organelle movement.

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