p34<sup>cdc2</sup>-mediated Phosphorylation Mobilizes Microtubule-organizing Centers from the Apical Intermediate Filament Scaffold in CACO-2 Epithelial Cells*

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The ability of simple epithelia to maintain a polarized phenotype is key to the function of various organs and glands in multicellular organisms. Both plasma membrane polarity (i.e. divided into apical and basolateral domains) and the polarized distribution of cytoplasmic organelles depend on the function of various components of the cytoskeleton (1, 2). The distribution of microtubules, oriented in the apico-basal axis with their minus ends under the apical domain, is believed to contribute to epithelial polarization (3). For example, the polarized distribution of the Golgi apparatus and the apical recycling endosomes (4) seems to depend on the polarized arrangement of microtubules. In turn, microtubules are organized in this peculiar fashion because centrosomes and non-centrosomal microtubule-organizing centers (MTOCs) are specifically localized to the apical cortical region (5–7). In fact, in polarized epithelial cells, centrosomes follow an interesting cycle: they localize under the apical domain in interphase but migrate to the lateral domain to form the mitotic spindle during mitosis (5). The organization of the spindle perpendicular to the apico-basal axis enables these cells to divide without loosing their polarity or disrupting the continuity of the epithelial barrier during mitosis (8). The advantage for the apical localization of centrosomes, on the other hand, is still unclear but may be related to the polarization of organelles and the potential to generate basal bodies in ciliated epithelia.

We have demonstrated that the apical localization of centrosomes depends on the distribution of intermediate filaments (IFs), which, in most simple epithelia, comprise heteropolymers of cytokeratins (CKs) 18 and 8 or 19 and 8. Centrosomes co-localize with apical IFs in epithelial cells, and down-regulation of IFs with antisense oligonucleotides results in changes of the position of centrosomes. Furthermore, we found co-immunoprecipitation of a specific MTOC component, γ-tubulin, and CKs, suggesting a true physical binding of MTOCs to IFs (7). The preliminary data in this work showed that in CACO-2 (human colon carcinoma) epithelial cells, a well-characterized system of polarized cells, IFs are stable during mitosis. This was surprising because IFs have been shown to undergo different types of reorganization during mitosis in some cell lines (9–11). However, this observation led to the hypothesis that centrosomes must detach from their IF anchors at the onset of mitosis to begin their migration to the lateral domain. Because our laboratory is keenly interested in identifying the molecular mechanisms involved in the attachment of centrosomes to IFs, this possibility offered a potential pathway to point to the molecule(s) that participates in this function (the “glue”). We reasoned that if centrosomes are indeed detached from IFs at the onset of mitosis, the trigger of the G2-M boundary, p34<sup>cdc2</sup> (cdk1)/cyclin B (12), must modify some of those molecules. The results show that a single protein, present in γ-tubulin/CK19 multiprotein insoluble complexes, is the target of p34<sup>cdc2</sup>-mediated phosphorylation, which can mobilize the centrosomes away from IFs even in interphase permeabilized cells.

EXPERIMENTAL PROCEDURES

CACO-2 human colon carcinoma epithelial cells were originally obtained from American Type Culture Collection (clone C2BBel, CRL-2102) and cultured as described previously (13). About 7 days after confluence, these cells differentiate and resemble polarized enterocytes (14). All experiments were performed on monolayers that had been confluent for 10–14 days. Metabolic labeling was done by incubating the cells in methionine/cysteine-free Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 30 μCi/ml [35S]methionine/cysteine (typically 1175 Ci/mmol; PerkinElmer Life Sciences). The antibodies used in this study were as follows: MPM-2 Mab anti-phospho-Ser/Thr-Pro (Upstate Biotechnology), polyclonal antibody anti-γ-tubulin (Sigma), Mab anti-CK18 (B23.1, DC 10; Biomeda Corp.), and Mab anti-CK19 (RCK108; Accurate Chemical & Scientific Corp.).

For cell permeabilization and p34<sup>cdc2</sup>-mediated phosphorylation, CACO-2 monolayers were washed in cold phosphate-buffered saline and permeabilized in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM EGTA, and 0.01% Brij supplemented with 1 mM ATP, 0.05% saponin,
and a mixture of antiproteases (Sigma P-8340) for 30 min. In some cases, 40 units/ml recombinant p34\(^{cd2}\) kinase and human cyclin B expressed in a baculovirus expression system and activated by cyclin-activating kinase phosphorylation (Biomol Research Laboratories Inc.) were added to the permeabilization buffer.

The procedures for immunofluorescence and confocal microscopy have been described elsewhere (7), as has the technique to obtain cytoskeletal fragments (13). Briefly, CACO-2 cell Triton X-100-insoluble pellets were obtained in the presence of an anti-protease mixture, homogenized, and extensively sonicated in a water\(\_\)ice system (4 hin total sonication, divided into 10-s sonication periods separated by 15-s periods to allow for heat dissipation). The fragments were separated on continuous sucrose gradients (20–60%) under rate velocity centrifugation conditions (27,600 \(\times\) g for 50 min). The top 4 ml of an 11-ml gradient were used for immunoprecipitation.

Immunoprecipitation and immunoblot were done as described previously (7). Phosphorylation with \(\gamma\)-\(\text{\textsuperscript{32}}\)P\(\text{ATP}\) was performed directly on multiprotein complexes immunoprecipitated with anti-\(\gamma\)-\(\text{\textsuperscript{32}}\text{~p}~\)tubulin antibody and still bound to the Sepharose beads via the antibody. The beads were incubated with the same buffer described above for cell permeabilization, except that cold ATP was replaced by 270 \(\mu\text{Ci/ml}\) \(\gamma\)-\(\text{\textsuperscript{32}}\)P\(\text{ATP}\) (PerkinElmer Life Sciences), and the same concentration of p34\(^{cd2}\) kinase and human cyclin B as described above for 30 min at room temperature. Negative controls were performed by omitting the kinase.

Quantification of signal in bands was done by acquiring digitized images of the chemiluminescence or radioactive emission (Multimage Light Cabinet from Alpha Innotech Corp. and Storm 840 from Molecular Dynamics, respectively) within the linear range of detection and weighting the size and intensity of a given band (number of pixels \(\times\) average intensity).

**RESULTS**

**Phosphorylation of Permeabilized CACO-2 Cell Monolayers with Recombinant p34\(^{cd2}\)**

**Results in Changes in the Localization of Centrosomes**—To achieve a rapid phosphorylation of p34\(^{cd2}\) targets in interphasic cells independently of the complex cascades that control the G\(_2\)-M boundary, we decided to directly introduce a recombinant, active form of the kinase after permeabilization of the plasma membrane. Because both centrosomes and IFs remain in a Triton X-100-insoluble fraction, it is unlikely that the “glue” proteins will be extracted by a mild permeabilization. To test the feasibility of this strategy, confluent CACO-2 epithelial cell monolayers were permeabilized with 0.05% saponin that, like digitonin, opens pores in the confluent CACO-2 epithelial cell monolayers were permeabilized except that cold ATP was replaced by 270 \(\mu\text{Ci/ml}\) \(\gamma\)-\(\text{\textsuperscript{32}}\text{~p}~\)P\(\text{ATP}\) and the same concentration of p34\(^{cd2}\) kinase and human cyclin B as described above for 30 min at room temperature. Negative controls were performed by omitting the kinase.

Because the IFs persist during mitosis in CACO-2 cells, and we demonstrated previously that morphological co-localization also represents physical attachment of centrosomes to IFs (7), we reasoned that the onset of mitosis must break such an attachment. To test this hypothesis, we phosphorylated intracellular targets of p34\(^{cd2}\) in saponin-permeabilized cells, as shown in Fig. 1. Permeabilization with saponin shrank the cells (evidenced by a decrease in the average length of the apico-basal axis), but in the absence of p34\(^{cd2}\) phosphorylation, it did not change the distribution of centrosomes (Fig. 2c). Nor did the incubation with nocodazole have any effect on the localization of centrosomes (Fig. 2e and g). However, upon phosphorylation with p34\(^{cd2}\), centrosomes separated from the cortical IFs were observed (Fig. 2d, arrow). When combined with nocodazole treatment, however, p34\(^{cd2}\) phosphorylation resulted in a substantial increase in the number of centrosomes isolated in the cytoplasm of interphasic cells (Fig. 2f, red channel, anti-CK18 antibody, compare with Fig. 1a; note that the magnification in Fig. 2b is \(2\times\) the magnification of the rest of the panels). The centrosomes were bigger than they were in interphase, and they were oriented in such a way that the mitotic spindle was roughly parallel to the plane of the monolayer (Fig. 2b, arrows, green channel, anti-\(\gamma\)-tubulin antibody), as described by others (8). Interestingly, however, unlike those in interphasic cells, the centrosomes in mitotic cells never co-localized with the cortical IFs (Fig. 2b, arrows).

![Fig. 1. Saponin-permeabilized CACO-2 cells can be phosphorylated with p34\(^{cd2}\). Confluent CACO-2 monolayers were permeabilized with saponin (a–f) and, in some cases, treated with p34\(^{cd2}\) kinase (e and f). All the cells were fixed, and control cells were treated with alkaline phosphatase (a and b). The monolayers were processed for immunofluorescence with the anti-\(\text{phospho-Ser/Thr-Pro monoclonal antibody MPM-2}\). b, d, and f are phase-contrast images of the same fields. Bar, 10 \(\mu\text{m}\).](image-url)
with exogenous recombinant p34\textsuperscript{cdc2}. This effect was enhanced by both anti-cytoskeletal drugs (Fig. 2, lined bars).

To confirm the morphological data, similar experiments were conducted on permeabilized CACO-2 monolayers, but the cells were scraped, sonicated, and extracted in Triton X-100. The distribution of \(\gamma\)-tubulin in the cytoskeletal pellet (p) and the supernatant (s) was analyzed by immunoblot (Fig. 3, top panel). In all cases, when the cells were not phosphorylated with p34\textsuperscript{cdc2}, the amount of soluble and insoluble \(\gamma\)-tubulin was similar, a result consistent with previous observations by other investigators (18). However, after p34\textsuperscript{cdc2} phosphorylation, the \(\gamma\)-tubulin bands associated with the cytoskeletal pellet were consistently smaller than those observed from the corresponding supernatants (Fig. 3, top panel, last six lanes). The same nitrocellulose sheets were reprobed with an anti-CK19 antibody as an internal control for the loading of the lanes (Fig. 3, bottom panel). Additionally, the CK19 immunoblot also confirmed the result shown in Fig. 16, namely, that p34\textsuperscript{cdc2} phosphorylation does not result in any significant depolymerization of IFs. To quantitatively assess these results, the bands from three experiments were measured by densitometry and normalized by calculating the ratio of signal in the pellet/supernatant in each condition. Confirming the morphological data, permeabilized cells not subjected to p34\textsuperscript{cdc2} phosphorylation showed similar distributions of \(\gamma\)-tubulin in the pellet (attached to the cytoskeleton) and the supernatant, disregarding the treatments with anti-cytoskeletal drugs (Fig. 3, graph, gray bars). However, when the cells were permeabilized and phosphorylated with p34\textsuperscript{cdc2} before extraction, the relative amount of cytoskeletal-bound \(\gamma\)-tubulin decreased significantly (Fig. 3, graph, striped bars). As observed in morphological experiments, this effect was also enhanced, to some extent, by anti-cytoskeletal drugs.

A Single Protein Is Phosphorylated by p34\textsuperscript{cdc2} in Multiprotein Fragments of the Insoluble Cytoskeleton in Which \(\gamma\)-Tubulin and CK19 Co-immunoprecipitate—Immunoprecipitation of IF-associated proteins is technically challenging because the Triton X-100-insoluble cytoskeleton co-pellets with agarose beads. To bypass this difficulty, in previous publications (7, 19) we developed a procedure to obtain multiprotein fragments of the Triton X-100-insoluble pellet, which can be used for immunoprecipitation, by extensive sonication. The assumption that these fragments represent a random sampling of the various insoluble multiprotein aggregates in the cells was proved correct at least for the main cytoskeletal components (7). In that publication, we demonstrated that \(\gamma\)-tubulin and CK19-8 co-immunoprecipitate in a subpopulation of these sonication fragments. To confirm those results and analyze the purity of these multiprotein fragments, we enhanced the published experiments by performing a sequential two-step immunoprecipitation with anti-\(\gamma\)-tubulin antibody first (as in the previous publication) and then with anti-CK19 antibody. Sonication fragments of the Triton X-100-insoluble pellet from metabolically labeled ([\(^{35}\text{S}\)]methionine/cysteine for 24 h) cells were subjected to this procedure with specific antibodies (Fig. 4A) or nonimmune IgG (Fig. 4B, control). The composition of these
immunoprecipitates was simpler than that seen when fragments were immunoprecipitated with only one antibody (data not shown; see Fig. 9 in Ref. 7), suggesting that a more pure subpopulation of multiprotein fragments had been isolated. In addition to the cytokeratin and \( /H9253\)-tubulin bands (in the 40–60 kDa range), only one additional major band was found (Fig. 4 A, arrow). Minor components of 220 and 90 kDa were only observed upon overexposure (data not shown). To analyze the effects of \( p34^{cdc2} \), the same immunoprecipitation of sonication fragments of the Triton X-100-insoluble pellet was repeated, starting from nonlabeled cells. After extensive washes, but before eluting the immunoprecipitates, the multiprotein complexes were subjected to \( p34^{cdc2} \)-mediated phosphorylation in the presence of \( /H9253-32P\)ATP. Only the high molecular mass protein was phosphorylated (Fig. 4 D, arrow) in an antibody- and \( p34^{cdc2} \)-specific manner (Fig. 4, C and E, controls). To confirm this result and determine the effect of \( p34^{cdc2} \)-mediated phosphorylation, similar experiments were repeated with three changes: (i) phosphorylation was performed in the presence of 1 mM cold ATP instead of tracer amounts of \( /gamma^{32P}\)ATP, (ii) phosphorylation was now assessed by the appearance of MPM-2 phospho-epitopes by immunoblot, and (iii) both immunoprecipitates and the trichloroacetic acid precipitate of the supernatant of the phosphorylation reaction were analyzed. The rationale for analyzing the supernatants was to determine whether any phosphorylated products were separated from the multiprotein complexes as a result of \( p34^{cdc2} \)-mediated phosphorylation. A band with the same apparent molecular mass as that observed with \( /H9253-32P\)ATP phosphorylation (Fig. 4 D) was found to be -come positive to MPM-2 and associated to the pellets, rather than the supernatants, after \( p34^{cdc2} \)-mediated phosphorylation (Fig. 4 J, another band of 65 kDa, positive for MPM-2 was found to be nonspecific because it appeared in negative controls as well). To determine whether the bands shown in Fig. 4, A, D, and J (arrows) represent the same peptide or are merely co-migrating proteins, a preparation of sonicated Triton X-100-insoluble fragments was obtained from \( [35S] \)methionine/cysteine-labeled cells (as in Fig. 4A), immunoprecipitated with anti-\( /gamma\)-tubulin antibody, and phosphorylated while still attached to the beads via the antibody with \( p34^{cdc2} \) and \( /gamma^{32P}\)ATP (as in Fig. 4D). The eluate was analyzed by two-dimensional gels and blotted. A short exposure of the blots, which does not suffice to see \( [35S] \)label, showed only two close spots, of the same apparent molecular mass as the band in Fig. 4D and pI = ~5 (Fig. 4N, arrow). The same membrane was extensively dephosphorylated, washed and exposed for 24 h to

![Graph](image-url)
**Phosphorylation Mobilizes MTOCs**

Cell permeabilization has been extensively used to allow access of extracellular reagents to intracellular targets (20–22). Both digitonin and saponin open pores in the membrane that allow the diffusion of large molecules, such as antibodies or enzymes, into the cytoplasm (15, 16, 23, 24). The results shown in this work indicate that in saponin-permeabilized cells, p34\(^{cdc2}\)-mediated phosphorylation does create MPM-2 epitopes at well known locations of the natural targets of this kinase (e.g. nucleus and centrosomes) and results in a 17-fold increase in the number of centrosomes that do not co-localize with cortical IFs (Figs. 1 and 2). These results were confirmed in independent biochemical studies (Fig. 3). Whereas nocodazole and cytochalasin D enhanced the effect of p34\(^{cdc2}\)-mediated detachment of centrosomes from the IF cortical lattice in vivo. A possible interpretation of the data is shown in Fig. 6.

**DISCUSSION**

Another preparation as described in lane D was obtained from \(^{35}\)S-methionine/cysteine-labeled cells, immunoprecipitated with anti-\(\gamma\)-tubulin antibody, and phosphorylated with p34\(^{cdc2}\) and \([\gamma-32P]ATP\). The eluate was analyzed in a two-dimensional gel (isoelectric focusing pH range 1–12 and SDS-PAGE). A 10-min exposure showed \(^{35}\)S-labeled peptides (N). After extensive dephosphorylation with alkaline phosphatase and washes, the same membrane was exposed for 24 h to show \(^{35}\)S-labeled proteins (O). Finally, the same membrane was reprophosphorylated in the presence of p34\(^{cdc2}\) and cold ATP and analyzed by immunoblot with MPM-2 Mab (P). In all cases, molecular mass standards are shown to the left in kDa.

**Fig. 4. A single protein that co-immunoprecipitates with \(\gamma\)-tubulin and CK19 is the target of p34\(^{cdc2}\)-mediated phosphorylation.** Lanes A and B, CACO-2 cells were metabolically labeled with \(^{[35}\)S\)methionine/cysteine for 24 h. Triton X-100-insoluble cytoskeletal pellets were extensively sonicated, and fragments useful for immunoprecipitation (0–1500 S) were separated in continuous sucrose gradients by rate velocity centrifugation. The fragments were immunoprecipitated with anti-\(\gamma\)-tubulin antibody. The immunoprecipitates were washed, eluted in 1% SDS, diluted five times in 1% Triton X-100 to allow renaturalization, and immunoprecipitated again with anti-CK19 antibody (A). An equal amount of protein was precipitated with the same sequential procedure but using nonimmune IgG of the same species in each step (B). Lanes C–E, sonication fragments of the Triton X-100-insoluble cytoskeletal pellet were divided into three identical aliquots and immunoprecipitated with anti-\(\gamma\)-tubulin antibody (C and D) or nonimmune IgG (E). While still attached to the protein A-agarose beads, the fragments were phosphorylated with p34\(^{cdc2}\) and \([\gamma-35\]P\)\(\)ATP. As a control for phosphorylation, p34\(^{cdc2}\) was omitted in one of the samples (C). Lanes F–M, an experiment similar to that shown in C and D was performed phosphorylating cytoskeletal sonication fragments immunoprecipitated on beads with anti-\(\gamma\)-tubulin antibody (F, G, J, and K) or nonimmune IgG (H, I, L, and M). Cold 1 mm ATP was used instead of \(^{32}\)P. p34\(^{cdc2}\) was omitted as a control in samples G, I, K, and M. In each case, the immunoprecipitates eluted from the beads (pellets, J–M) or the trichloroacetic acid precipitates from the corresponding supernatants (F–I) were analyzed by immunoblot with MPM-2 Mab. N–P, display \(^{35}\)S label, showing that the preparation comprises different peptides (Fig. 4O). Finally, the same membrane was reprophosphorylated in the presence of p34\(^{cdc2}\) and cold ATP and analyzed with MPM-2 Mab and chemiluminescence (as in Fig. 4J). In this case, only the high molecular mass doublet was positive (Fig. 4P, arrow), suggesting that the –65-kDa band in Fig. 4, F–M, does not have a true cdc2-specific phosphorylation site. These results suggest that the target of p34\(^{cdc2}\) in the Triton X-100-insoluble sonication fragments remains attached to the \(\gamma\)-tubulin side of the complexes after phosphorylation. In addition, the doublet of spots of identical molecular mass suggests that the peptide shown in Fig. 4D may have two cdc2 phosphorylation sites (Fig. 4, N and P, arrows). CK19 Is Separated from \(\gamma\)-Tubulin-containing Insoluble Multiprotein Complexes after p34\(^{cdc2}\) Phosphorylation—Then the question arose of whether p34\(^{cdc2}\)-mediated phosphorylation of one component of the multiprotein complexes that contain \(\gamma\)-tubulin and CK19 suffices to separate the latter from the complexes. A set of experiments similar to that described in Fig. 4, F–M, was performed. This time, the pellets (immunoprecipitates) and trichloroacetic acid precipitates of the supernatants of the phosphorylation reaction were analyzed by immunoblot with an anti-CK19 antibody (Fig. 5, top panel). Because the initial amount of protein at the beginning of the immunoprecipitation was carefully normalized, we also measured the signal of each positive band and the average relative intensities of specific bands, normalized as the percentage of the band in the untreated pellet (Fig. 5, bottom panel). It was found that the amount of CK19 attached to \(\gamma\)-tubulin-containing complexes decreased as a result of p34\(^{cdc2}\)-mediated phosphorylation (Fig. 5, lane A versus lane B). More importantly a band approximately representing the amount of CK19 missing from the pellets (signal in lane B – signal in lane A, or 100% – the value in the left-hand side bar in the bottom panel) appeared in the supernatants only in the case of p34\(^{cdc2}\)-mediated phosphorylation (Fig. 5E). This result suggests that the multiprotein complexes that contain both \(\gamma\)-tubulin and CK19 also suffer a physical separation of the latter after p34\(^{cdc2}\)-phosphorylation, mimicking the in vitro detachment of centrosomes from the IF cortical lattice in vivo. A possible interpretation of the data is shown in Fig. 6.
Phosphorylation Mobilizes MTOCs

Phosphorylation in separating centrosomes from IFs, it is worthwhile to note that in any case, neither of the anti-cytoskeletal drugs had any effect on centrosome distribution per se. This confirms previous observations in our laboratory (7) and leads us to conclude that p34cdc2-mediated phosphorylation is a prerequisite for the mobilization of centrosomes. Once the centrosomes are released from their anchoring structure (presumably IFs) at the onset of mitosis (or by p34cdc2-mediated phosphorylation), microtubules and actin filaments seem to be important to mobilize them or hold them at a certain region during mitosis, as suggested by others (25). In other words, we propose a two-step process: first, release from the IFs, and second, positioning the centrosomes and the mitotic spindle by interactions between astral microtubules, perhaps cortical actin, and peripheral cues (8, 25). The results in this work highlight the occurrence of the first step. Alternatively, the effect of nocodazole and cytochalasin D in enhancing the effect of p34cdc2-mediated phosphorylation may be explained in more simple terms as a consequence of nonspecific retention of rather big structures (i.e. centrosomes) in a meshwork-like structure (actin or tubulin cytoskeleton) that is depolymerized by these drugs. Unfortunately, the data presented here do not allow us to distinguish between these two possibilities, and this type of analysis is beyond the scope of this work. It has been known for two decades that cytokeratins disorganize during mitosis in some 50% of epithelial cell lines. To our knowledge, there are still no explanations as to why IFs are stable in some cell lines, but cytokeratins in cell lines from stratified epithelia seem to be more prone to be disorganized during mitosis than those in cell lines originating from simple epithelia (26). The general biological implications of the of p34cdc2-mediated detachment of MTOCs from keratin IFs described here will depend on the behavior of keratin IFs during mitosis in vivo, which, to our knowledge, is still poorly understood.

In previous publications, we resorted to isolation of relatively small multiprotein aggregates obtained by sonication to be able to perform co-immunoprecipitation (or pull-down) experiments from insoluble cytoskeletal preparations otherwise not amenable for those procedures, while preserving protein-protein interactions (7, 13, 19). Although it is likely that the complexes isolated by double immunoprecipitation (anti-γ-tubulin and anti-CK19) by this procedure represent part of the centrosome-IF interphase ("glue"), the available data do not allow us to fully equate them to any specific microdomain of the cytoskeleton. For example, a large number of small noncentrosomal MTOCs are present in epithelial cells (6) and are also apically localized as well as centrosomes (6, 7). At this point, it is not possible to assert whether these fragments represent at least part of the molecules involved in the attachment of noncentrosomal MTOCs, centrosomes, or both. It was reassuring to learn, however, that these insoluble multiprotein aggregates respond to p34cdc2-mediated phosphorylation as expected from the true interphase between MTOCs and IFs, by splitting the IF part (that contains CK19) away from the MTOC part (that includes γ-tubulin) (Figs. 5 and 6). One of the original goals of this work was to identify proteins that would be candidates for the role of "glue" between MTOCs and IFs. In this regard, the single target for p34cdc2-mediated phosphorylation found in the immunoprecipitated sonication fragments (Fig. 4) seems to fulfill some of the conditions expected for such a molecule. Unfortunately, none of the centrosomal components known to us displaying a molecular mass in the 160–220 kDa range contain a consensus phosphorylation site for p34cdc2. Plectin, an IF-associated protein, is a target of p34cdc2 and mediates mitotic changes in the organization of IFs (27). However, plectin has a much higher molecular mass and would be expected to remain associated with IF proteins and not with the MTOC part, as we observed here (Fig. 4). In addition, plectin displays a characteristic basal distribution (28) as opposed to the typical apical distribution of MTOCs. Therefore, the molecular identity of the target of p34cdc2 described in this work remains elusive. Our laboratory is currently engaged in a long-term effort to purify...
micromolar amounts of this protein to identify it by endoprotease digestion and mass spectroscopy. Although the mechanistic role of this protein cannot be determined until the sequence is available, a very preliminary model to interpret the data in this work is shown in Fig. 6. Assuming that the sonication fragments provide a sampling of the interphase between intermediate filaments and MTOCs, they may contain also IF-associated proteins. One peptide in these complexes is phosphorylated by p34<sup>cdc2</sup>, possibly in two sites (Fig. 4, N and P), and thus mediates the dislodging of MTOCs from IFs. The precise map of protein-protein interactions in the MTOC-IF interphase remains to be determined. However, the results reported here suggest that this as yet unidentified target of p34<sup>cdc2</sup> may play a critical role in the localization of MTOCs in epithelial cells in interphase and thus in the polarized orientation of microtubules.

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REFERENCES
1. Nelson, W. J. (1992) Science 258, 948–955
2. Yeaman, C., Grindstaff, K. K., Hansen, M. D., and Nelson, W. J. (1999) Curr. Biol. 9, R515–R517
3. Bacia, R., Antony, C., Dotti, C., Karsenti, E., Stelzer, E. H. K., and Simons, K (1989) J. Cell Biol. 109, 2817–2832
4. Apodaca, G., Katz, L. A., and Mostov, K. E. (1994) J. Cell Biol. 125, 67–86
5. Buendia, B., Bre, M. H., Griffiths, G., and Karsenti, E. (1990) J. Cell Biol. 110, 1123–1135
6. Meads, T., and Schroer, T. A. (1995) Cell Motil. Cytoskeleton 32, 273–288
7. Salas, P. J. I. (1999) J. Cell Biol. 14, 645–657
8. Reinoso, S., and Karsenti, E. (1994) J. Cell Biol. 126, 1509–1526
9. Foisner, R. (1997) Bioessays 19, 297–305
10. Franke, W. W., Schmid, E., Grund, C., and Geiger, B. (1982) Cell 30, 103–113
11. Chou, C.-F., and Omary, M. B. (1993) J. Biol. Chem. 268, 4465–4472
12. Ashertan-Peeler, S., Parker, L. L., Geahlen, R. L., and Piwnica-Worms, H. (1993) Mol. Cell. Biol. 13, 1675–1685
13. Salas, P. J. I., Rodriguez, M. L., Viciana, A., Vega-Salas, D. E., and Hauri, H. P. (1997) J. Cell Biol. 137, 359–375
14. Pinto, M., Robine-Leon, S., Appay, M.-D., Kedinger, M., Triadou, N., Dussaux, E., Laceix, B., Simon-Assmann, P., Haffen, K., Fogh, J., and Zweibaum, A. (1983) Biol. Cell 47, 323–330
15. Schulz, I. (1990) Methods Enzymol. 192, 280–300
16. Lepers, A., Cacan, R., and Verbert, A. (1990) Biochimie (Paris) 72, 1–5
17. Melan, M. A. (1994) Methods Mol. Biol. 34, 55–66
18. Stearns, T., and Kirschner, M. (1994) Cell 76, 623–637
19. Rodriguez, M. L., Brignoni, M., and Salas, P. J. I. (1994) J. Cell Sci. 107, 3145–3151
20. Gonzalez, C. A., Gottfried, C., and Dunkley, P. R. (2000) Neurochem. Res. 25, 885–894
21. Liu, J., Xiao, N., and DeFranco, D. B. (1999) Methods (Duluth) 19, 403–409
22. Hersey, S. J., and Perez, A. (1999) Annu. Rev. Physiol. 52, 345–361
23. Tang, B. L., Tan, A. E., Lim, L. K., Lee, S. S., Low, D. Y., and Hong, W. (1998). J. Biol. Chem. 273, 6944–6950
24. Csala, M., Banhegyi, G., Braun, L., Szirmai, R., Burchell, A., Burchell, B., Benedetti, A., and Mandl, J. (2000) Biochem. Pharmacol. 59, 801–805
25. Busson, S., Dujardin, D., Moreau, A., Dompierre, J., and De Mey, J. R. (1998) Curr. Biol. 8, 541–544
26. Lane, E. B., Goodman, S. L., and Trejos-Siewiec, I. K. (1982) EMBO J. 1, 1365–1372
27. Foisner, R., Malecz, N. Dressel, N., Stadler, C., and Wiche, G. (1996) Mol. Biol. Cell 7, 273–298
28. Hieda, Y., Nishizawa, Y., Uematsu, J., and Owaribe, K. (1992) J. Cell Biol. 116, 1497–1506

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