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Mutations in the kinesin-like protein Eg5 disrupting localization to the mitotic spindle

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ABSTRACT **Eg5, a member of the bimC subfamily of kinesin-like microtubule motor proteins, localizes to spindle microtubules in mitosis but not to interphase microtubules. We investigated the molecular basis for spindle localization by transient transfection of *Xenopus* A6 cells with myc-tagged derivatives of Eg5. Expressed at constitutively high levels from a cytomegalovirus promoter, mycEg5 protein is cytoplasmic throughout interphase, begins to bind microtubules in early prophase, and remains localized to spindle and/or midbody microtubules through mitosis to the end of telophase. Both N- and C-terminal regions of Eg5 are required for this cell-cycle-regulated targeting. Eg5 also contains within its C-terminal domain a sequence conserved among bimC subfamily proteins that includes a potential $p34^{cdc2}$ phosphorylation site. We show that mutation of a single threonine (T937) within this site to nonphosphorylatable alanine abolishes localization of the mutant protein to the spindle, whereas mutation of T937 to serine preserves spindle localization. We hypothesize that phosphorylation of Eg5 may regulate its localization to the spindle in the cell cycle.**

Microtubule motor proteins, most notably members of the kinesin gene family (1), have been implicated in various intracellular processes, including vesicle transport, mitotic movements, and the organization of microtubule-based structures (2–6). How specific motor proteins are targeted and/or regulated to perform specific biological functions remains, however, largely unknown. Because members of the kinesin family share common sequences within a conserved motor domain, or "head," it is presumed that functional specificity is determined primarily by nonconserved, nonmotor "tail" domains (6). Tail domains could be expected to regulate motor activity or localization in any number of ways, such as interacting with head domains to effect conformational changes in protein structure (7, 8), or binding to additional nonmotor proteins with specific intracellular locations (9, 10); other mechanisms of regulation are equally possible. Clearly, in order to understand motor protein function *in vivo*, a considerable importance is attached to identifying modes of motor protein regulation and targeting.

We previously demonstrated that the kinesin-like protein Eg5 of *Xenopus laevis* (11) is a plus-end-directed microtubule motor protein *in vitro* and is required for efficient spindle formation in an *in vitro* assembly reaction (12, 13). Relatives of Eg5 have been identified in a number of different organisms and, together with Eg5, these genes—bimC in *Aspergillus nidulans* (14, 15), cut7 in *Schizosaccharomyces pombe* (16), KLP61F in *Drosophila melanogaster* (17), and CIN8 and KIP1 in *Saccharomyces cerevesiae* (18, 19)—appear to define a subfamily of kinesin-like proteins (the "bimC subfamily"). All are involved in the assembly and/or function of the spindle and, where determined, spindle-associated *in vivo* (13, 18, 20). The bimC-like proteins are thought to be similar in overall structure, having an N-terminal motor domain followed by a region of predicted α-helix and a nonhelical tail domain of yet unknown function. Within the motor domain, amino acid sequences of bimC subfamily proteins are more similar to each other than to those of other kinesin-like proteins (1). Conservation of amino acid sequence outside the motor domain, however, is limited to an ≈40-residue region in the tail domain that is at least partially conserved in all subfamily members except CIN8 and KIP1. Heck *et al.* (17) have suggested that this region, which we shall refer to as the "bimC box," may act as a functional specificity determinant. The most highly conserved portion of the bimC box includes the sequence TGX-TPXK/RR (in Eg5 it is TGTTPQRR), and it has been noted (17) that this sequence could be phosphorylated by a serine (threonine) protein kinase such as the proline-directed kinase (21) or an ERK-family kinase (22, 23). We have further noticed that this same sequence also includes the consensus phosphorylation site for the cell-cycle kinase $p34^{cdc2}$ (consensus S/TPXK/R) (24). As yet, however, no evidence for phosphorylation of the bimC box by any serine(threonine) protein kinase has been obtained.

To understand the mechanism by which Eg5 and related proteins work in mitosis, we would like to know (*i*) how they localize to the spindle, (*ii*) with what other spindle components they interact, and (*iii*) how their activity is regulated throughout the cell cycle and through the different stages of mitosis. Here we address the first of these questions, by mutating regions of the Eg5 protein that may be involved in targeting it to the spindle. We have found that elements of both the N- and C-terminal domains of Eg5 are required for spindle localization. Interestingly, a single point mutation in the most conserved part of the bimC box is able to abolish spindle localization completely, in a manner that is consistent with phosphorylation regulating the localization and/or activity of Eg5.

MATERIALS AND METHODS

Plasmid Construction. All constructs (Fig. 1) were made using the Eg5K2 gene (25), which is >90% identical to the Eg5 gene originally described (now termed Eg5K1; see ref. 11) and thought to be the result of pseudotetraploidy in *Xenopus*. A 3.2-kb near-full-length fragment of Eg5K2 was generated by partial *Eco*RI digestion of a cDNA clone in Bluescript II KS+. This fragment does not encode the N-terminal amino acids MSSQNSFMSSKKDD (the translational start site may be at either Met) but does encode all remaining residues (15–1067; our numbering scheme assumes translation initiation at the first Met; ref. 25). The initial amino acids are dispensable for motility *in vitro* (13) as well as for localization *in vivo* (see *Results*). The myc-tagged "near-full-length" plasmid pEM103 was constructed by cloning the 3.2-kb *Eco*RI fragment into the *Eco*RI site of mycHB, a vector derived from pcDNAI containing a cytomegalovirus promoter and an N-terminal c-myc

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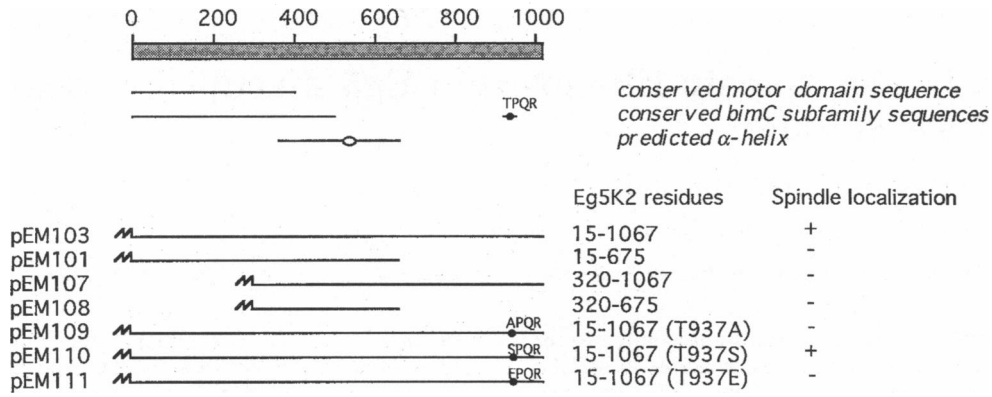

FIG. 1. (*Upper*) Schematic of Eg5 coding sequence. Amino acid residue numbers are shown above, and properties of the coding sequence are shown below. "TPQR" indicates the potential p34cdc2 phosphorylation site in the C-terminal portion (bimC box; see text) of the conserved bimC subfamily sequences. The bubble in the predicted α-helix indicates a short region of predicted nonhelix. (*Lower*) Names of plasmids encoding mycEg5K2 and various derivatives, the actual residues encoded (the jagged line represents the myc tag), and whether the encoded proteins localize to the spindle in mitosis (see text and subsequent figures).

epitope (see below). The mycHB plasmid was the gift of P. Garcia (University of California, San Francisco). The C-terminal truncation pEM101 was made by cloning the N-terminal *Eco*RI fragment of the Eg5K2 cDNA (residues 15–675) into the same site of mycHB. The polypeptide sequence upstream of the Eg5K2 coding sequence for both pEM101 and pEM103 is MTAEQKLISEEDLNGGRVQDPLVTAASVLEFR. In addition, pEM101 and pEM108 (see below) encode the 26 amino acids FCRYPSHWRPLEHASRGPYSIVSPKC at their C termini as a consequence of the construction. The N-terminal truncation pEM107 was made by digesting pEM103 with *Bam*HI, which cuts just 5′ to the *Eco*RI site of mycHB and at residue 319 in the Eg5K2 coding sequence. The *Bam*HI digest was subsequently treated with mung-bean nuclease to put the cDNA in frame with the myc tag upon ligation/recircularization of the plasmid; this was confirmed by the creation of a new *Pvu* II site at the joint. The double N/C-terminal deletion pEM108 was created by removing the comparable bases from pEM101. The polypeptide sequence upstream of Eg5K2 coding regions in pEM107 and pEM108 is MTAEQKLISEEDLNGGRVQ, followed by Eg5K2 residues 320–1067 (pEM107) and Eg5K2 residues 320–675 (pEM108).

Site-directed mutations at T937 of Eg5K2 were created by PCR (26), amplifying a region from an *Acc* I site at residue 922 to a *Xho* I site 3′ to the Eg5K2 coding region. Amplified fragments were cloned into a *Xho* I/partial *Acc* I digest of pEM103. The nucleotide sequence for residues 936–937 in pEM103 is ACA ACG. In pEM109 (T937A) it is ACA GCG; in pEM110 (T937S) it is ACG TCG; and in pEM111 (T937E) it is ACA GAG. All mutants were confirmed by sequencing within the amplified region, at which time we discovered that the sequence of our original Eg5K2 cDNA was different from the published sequence (25) in four positions. At position 951 we found the codon AGA (Arg) instead of AAA (Lys); at 963, AAG (Lys) instead of AGG (Arg); at 1020, GGC (Gly) instead of GGG (Gly); and at 1038, GCA (Ala) instead of GGA (Gly). The reasons for these small discrepancies are not yet clear. Given that all of these differences are silent or conservative and are present in all of our constructs, we do not believe that this affects our conclusions.

Cell Culture and Transfections. *X. laevis* A6 cells were grown on tissue culture dishes in 70% Leibovitz's L-15/15% fetal calf serum supplemented with penicillin/streptomycin and glutamine. Cells were grown to subconfluence in 60-mm plastic dishes and transfected with Qiagen-purified plasmid DNA using the calcium phosphate method (27), except that Hanks' balanced salts contained lower NaCl (1× = 100 mM) to correct for the lower tonicity of *Xenopus* culture medium. Fifteen micrograms of plasmid DNA without carrier was used per 60-mm culture dish. Cells were exposed to calcium phosphate/DNA coprecipitate for 5–6 hr and then washed extensively in 0.7× phosphate-buffered saline (PBS; with calcium/magnesium) before being returned to fresh medium. Glycerol shock was not advantageous in this instance, for while it increased transfection effiency about 2- to 3-fold, the percentage of mitotic cells 48 hr after transfection (see below) was considerably reduced.

Immunofluorescence. Individual 60-mm dishes were washed with calcium/magnesium-free 0.7× PBS 20–24 hr posttransfection, trypsinized, and resuspended in 0.5–1.0 ml of fresh medium and then plated onto one or two 18-mm square coverslips that had been previously acid-washed and poly(L-lysine)-coated, and further coated with Matrigel (Collaborative Research) just before use. Thirty-six to 48 hr posttransfection, coverslips were rinsed in 0.7× PBS and fixed in cold methanol. Cells were then rinsed in Tris-buffered saline (TBS)/0.1% Triton X-100 (TBS/TX) and processed for immunofluorescence, using a hybridoma supernatant of the anti-myc antibody 9E10, which recognizes the sequence EQKLISEEDLN (28). Because nonspecific background staining by 9E10 was particularly high in A6 cells, the blocking medium and antibody incubation medium used for 9E10 staining was TBS/TX containing 3% ovalbumin (Sigma grade V) and 0.5 M NaCl. For all other immunofluorescence the blocking solution and antibody diluent was TBS/TX/2% bovine serum albumin. An anti-Eg5 antibody ("AST") was raised against a glutathione *S*-transferase fusion protein containing Eg5K2 residues 320–1067 and affinity-purified on the same protein bound to Affi-Gel-10 (Bio-Rad) after removal of anti-glutathione *S*-transferase antibodies as described (29). AST was used in immunofluorescence experiments at 20 μg/ml. The anti-tubulin antibody DM1α (Amersham) was used as directed by the manufacturer. Secondary antibodies were from Jackson Immunoresearch. Cells were counterstained with 1 μg of propidium iodide per ml where appropriate and mounted in Tris-buffered glycerol containing 1% phenylene diamine as an anti-fade ingredient.

Cells expressing the mycEg5 proteins were identified by scanning stained coverslips with a 25×/0.8 numerical aperture multi-immersion objective (Zeiss) and a dual-channel fluorescein/rhodamine filter set (Chroma Technology, Brattleboro, VT) mounted on a Photomicroscope III (Zeiss). A typical transfection of ≈8 × 10^5 cells yielded ≈50–100 that expressed the mycEg5 construct above background staining and were in mitosis as judged by propidium iodide staining of chromosomes. This reflects a typical transfection efficiency of 1.5–2.0% and a mitotic index of about 0.5–1.0% under the condi-

tions of the experiment. In each experiment at least 35 cells were scored, and in all experiments the localizations described were observed in 100% of the cells. Z-series optical sections of representative cells were collected with a Bio-Rad MRC-600 confocal microscope and projected to produce the images shown in Figs. 2–4.

RESULTS

We previously demonstrated that Eg5 is present in spindles assembled *in vitro* in *Xenopus* egg extracts and is particularly enriched near spindle poles (13). To investigate possible cell-cycle regulation of Eg5 localization we examined its distribution in the *Xenopus* cell lines A6, XL177, and XTC (30) by indirect immunofluorescence. The antibody used (see *Materials and Methods*) was highly specific for Eg5 on Western blots of whole-cell lysates made in Laemmli buffer (ref. 13 and additional data not shown), and the staining patterns observed were identical in all three cell types; staining in A6 cells is described further below.

In interphase cells we observed a weak, diffuse cytoplasmic staining that was only slightly detectable above background (Fig. 2*A*). However, by early prophase (i.e., prior to nuclear envelope breakdown and reorganization of the interphase microtubule array), Eg5 staining appeared very prominently along microtubules close to the centrosome and to a lesser extent on more peripheral microtubules (Fig. 2*B*; see also Fig. 3*B*). At later stages of mitosis Eg5 was strongly localized to mitotic spindle microtubules, and this staining persisted through the end of mitosis, although astral microtubules remained unstained (Fig. 2*C*). In contrast to our previous observations of spindles assembled *in vitro* (13), we did not find Eg5 to be highly enriched at spindle poles at metaphase, although a more polar distribution was readily apparent at both earlier and later stages of mitosis (see Fig. 3). A generally similar pattern of Eg5 staining has also been described in A6 cells by Houliston *et al.* (25), although they observed a more polar localization throughout all stages of mitosis.

We expressed a derivative of Eg5 tagged with an N-terminal c-myc epitope (mycEg5K2/15–1067; Fig. 1) in A6 cells by transient transfection. Like the endogenous Eg5, the myc-tagged Eg5 was spindle-associated in mitosis but exclusively cytoplasmic during interphase, even when expressed at very high levels (Fig. 3). A fraction of mycEg5K2/15–1067 protein remained cytoplasmic during mitosis (perhaps as a consequence of saturated binding sites), but this did not have any obvious ill effects on mitotic progression, as judged by the relative proportions of late-stage mitotic cells and respreading sister cells. These results suggest that Eg5 localization to microtubules is tightly regulated in the cell cycle.

To investigate which domains of Eg5 might be responsible for regulating its localization, we expressed N- and C-terminal fragments of Eg5 containing the myc epitope (mycEg5K2/15–675 and mycEg5K2/320–1067, respectively; see Fig. 1). Both N- and C-terminal fragments failed to localize to the spindle in mitosis, as did the double N- and C-terminal truncation mycEg5K2/320–675 (Fig. 1), although all three proteins were clearly expressed at high levels. Representative staining (of mycEg5K2/15–675) is shown in Fig. 4 *A* and *B*. These results suggest that both N- and C-terminal regions of Eg5 are involved in spindle localization.

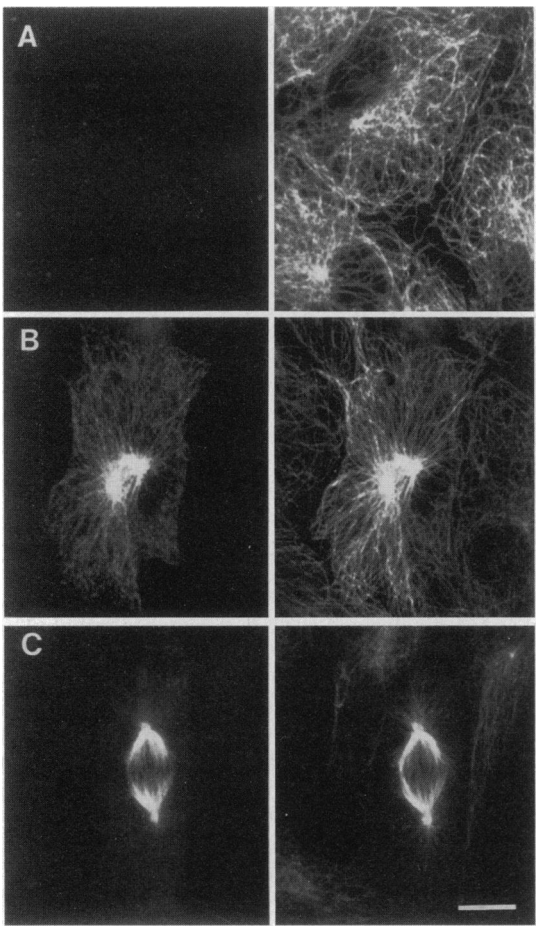

FIG. 2. Localization of endogenous Eg5 in *Xenopus* A6 cells. Paired images of anti-Eg5 (*Left*) and anti-tubulin (*Right*) staining. (*A*) Interphase. (*B*) Prophase. (*C*) Metaphase. (Bar = 10 μm.)

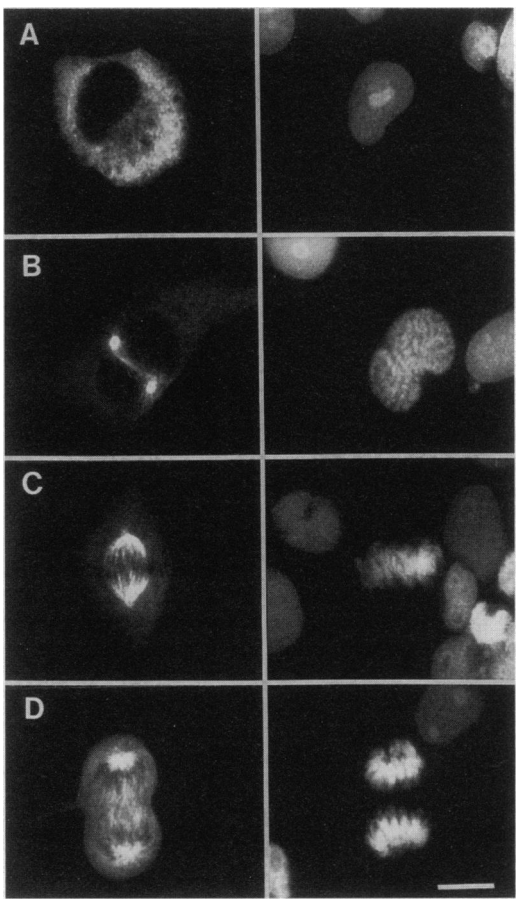

FIG. 3. Localization of mycEg5K2/15–1067 in transfected A6 cells. Paired images of anti-myc (*Left*) and propidium iodide (*Right*) staining. (*A*) Interphase. (*B*) Prophase. (*C*) Metaphase. (*D*) Late anaphase. (Bar = 10 μm.)

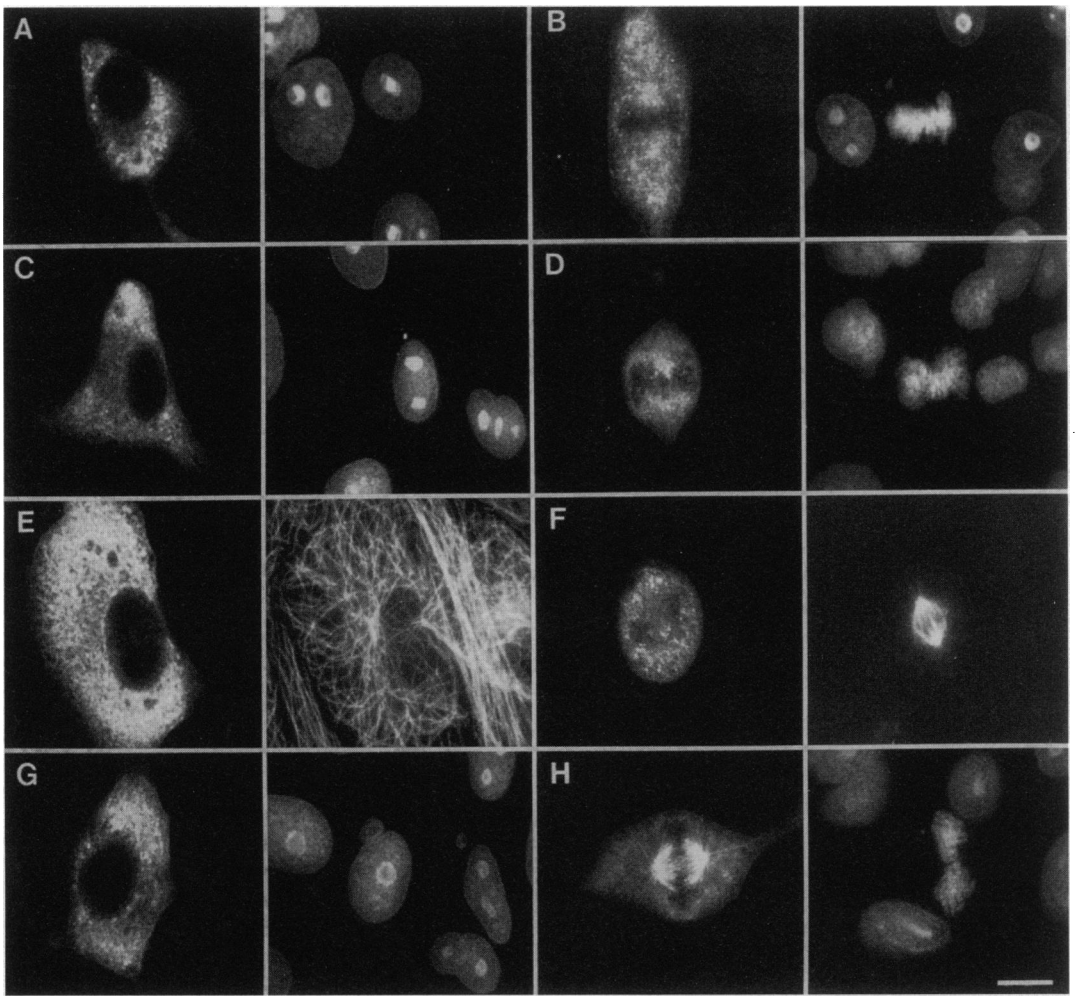

FIG. 4. Localization of mycEg5K2 derivatives in transfected A6 cells. Paired images of anti-myc (*Left*) and propidium iodide (*Right*) staining (*A–D, G,* and *H*) or anti-myc (*Left*) and anti-tubulin (*Right*) staining (*E* and *F*). Cells in *A, C, E,* and *G* are in interphase; cells in *B, D, F,* and *H* are in metaphase. (*A* and *B*) mycEg5K2/15–675. (*C–F*) mycEg5K2/15–1067(T937A). (*G* and *H*) mycEg5K2/15–1067(T937S). (Bar = 10 μm.)

We were also interested in the function of the bimC box in regulating Eg5 localization and, in particular, whether a special role might be played by T937 (the third threonine residue in the sequence TGTTPQRR), which is most likely to be phosphorylated by $p34^{cdc2}$ or an ERK kinase. When T937 was mutated to alanine by site-directed mutagenesis [mycEg5K2/15–1067(T937A); Fig. 1], spindle localization was completely abolished, and the expressed protein remained in the cytoplasm (Fig. 4 *C* and *D*). As with the truncation mutants, there were no obvious deleterious consequences of mutant expression. Cells expressing the T937A mutation were observed in all stages of mitosis and displayed normal interphase and mitotic microtubule arrays when doubly stained with anti-myc and anti-tubulin antibodies (Fig. 4 *E* and *F*). We then mutated T937 to serine, which might be expected to preserve spindle localization if phosphorylation by a serine(threonine) kinase were involved in localization. The mutant protein mycEg5K2/15–1067(T937S) (Fig. 1) was cytoplasmic during interphase and spindle-associated in mitosis, indistinguishable from mycEg5K2/15–1067 and the endogenous protein (Fig. 4 *G* and *H*). These experiments indicate that T937 is indeed critical for determining Eg5 localization and further suggest that localization may be regulated by a serine(threonine) kinase that modifies T937. As a further test we also mutated T937 to glutamate, as mutation of threonine or serine to glutamate can occasionally mimic a phosphorylated residue without perturbing biological function (ref. 31, but see also refs. 32–34). The mutant protein mycEg5K2/15–1067(T937E) (Fig. 1) did not, however, localize to the spindle but rather behaved identically to the T937A mutant (not shown). Again we did not observe any gross effects on mitosis, although anecdotally we observed perhaps a higher proportion of prometaphase cells, which might indicate a brief pause in mitotic progression.

DISCUSSION

Our results suggest that a discrete switch regulates Eg5 localization during the cell cycle. Endogenous Eg5 in *Xenopus* A6 cells is bound to microtubules in mitosis but not during interphase. A similar cell-cycle-dependent microtubule association for Eg5 has been observed by Houliston *et al.* (25), who also found that Eg5 may be associated with interphase microtubules during early *Xenopus* development. The faint staining seen in interphase A6 cells may indicate that total levels of Eg5 could be somewhat lower in interphase relative to mitosis; however, we also found that a myc-tagged Eg5 was expressed at high levels at all stages of the cell cycle and nonetheless localized to microtubules only in mitosis. We further note that the localization of Eg5 to prospective spindle poles at prophase, prior to nuclear envelope breakdown and the reorganization of the interphase microtubules into a bipolar spindle, suggests that Eg5 localization to spindle microtubules is perhaps likely to be controlled more directly by cell-cycle regulatory machinery than by binding to other spindle-

associated components. More generally, this polar localization suggests that microtubule populations within a single cell can be biochemically and probably dynamically distinct even at the very earliest stages of mitosis. Such differences, exemplified in later stages of mitosis by the formation of kinetochore fibers, are likely to be important in the assembly of the mitotic spindle.

What can we say about the molecular mechanisms governing the localization of Eg5? A simple deletion analysis indicates that both N- and C-terminal regions are required for spindle localization and microtubule binding in mitosis. While we previously showed that an N-terminal fragment of Eg5 identical to that of mycEg5K2/15–675 is able to bind and translocate microtubules *in vitro* when fused to glutathione *S*-transferase and purified from *Escherichia coli* (13), it may not be unreasonable to suspect that a similar fragment might not bind microtubules strongly in the presence of intracellular concentrations of ATP. However, if this is the case we must nevertheless explain how the C terminus of Eg5 is involved in promoting microtubule binding. In contrast to the C terminus of kinesin, which does localize to microtubules when expressed independently in mammalian cells (35), the C terminus of Eg5 does not bind microtubules on its own and thus would appear not to have any distinct "spindle targeting signal" (assuming the tagged proteins fold properly *in vivo*). We believe that the simplest interpretation of our results is that (*i*) microtubule binding and spindle localization are indeed mediated directly through the N-terminal motor domain, but, in addition, (*ii*) an intact C terminus, itself incapable of binding microtubules, is further required for strong microtubule binding *in vivo*. One possible explanation for this behavior might be that as yet unidentified cellular proteins repress the activity of Eg5 *in vivo* by binding or modifying its motor domain, while the tail domain abrogates this inhibition, perhaps in a cell-cycle-regulated manner. This interpretation implies that conformational interactions between motor and nonmotor domains of Eg5 are likely to be essential for proper function *in vivo*. These notions are particularly interesting in light of recent experiments suggesting that a likely Eg5 homolog in *Drosophila* embryos can be isolated as a homotetramer (36); one could thus speculate, for example, that Eg5 is functionally active *in vivo* only in higher-order structures that require an intact C terminus for their assembly. We note that neither our present experiments nor our interpretation of them, however, can address the question of how Eg5 is enriched at spindle poles or why Eg5 is not observed on astral microtubules.

Most strikingly, we have further found that at least one of the critical C-terminal elements governing spindle localization is T937. Our observation that T937 can be mutated to serine without effect but not to alanine is consistent with an important function for a hydroxyl group at this position. Given its location in a consensus phosphorylation site for a number of protein kinases, including the mitotic regulator $p34^{cdc2}$, it seems reasonable to hypothesize that the cell-cycle-regulated localization of Eg5 may in part result from a cell-cycle-dependent phosphorylation of T937. Recently it has been shown that a C-terminal, nonmotor, microtubule binding domain of the kinesin-like kinetochore protein CENP-E may be regulated through phosphorylation by $p34^{cdc2}$ (37). While we do not yet know if Eg5 is indeed phosphorylated *in vivo*, future work combining mutant Eg5 proteins with *Xenopus* egg extracts should allow for a detailed analysis of Eg5 phosphorylation in the cell cycle as well as the identification of potential interacting proteins and/or mechanisms that may modify the localization or activity of Eg5 *in vivo*.

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