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Original article

The *Xenopus laevis* centrosome aurora/Ipl1-related kinase

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The cDNA encoding the protein kinase pEg2 was originally cloned through a differential screening performed during the early development of *Xenopus laevis*. pEg2 orthologues were found in various organisms and were classified in a new family of oncogenic mitotic protein kinases named 'aurora/Ipl1-related kinases' after the *Drosophila melanogaster* gene *aurora* and the *Saccharomyces cerevisiae* gene Ipl1. The catalytic activity of pEg2 is necessary for the mitotic microtubule spindle formation in *Xenopus laevis* egg extracts. The addition of a dominant negative form of pEg2 to *in vitro* spindle assembly assays leads to monopolar spindles generated by a defect of centrosome separation. In *Xenopus* cultured cells, pEg2 was confined around the pericentriolar material once centrosomes were duplicated. The centrosome localization does not depend on the presence of microtubules. However, *in vitro*, the protein binds to taxol-stabilized microtubules independently of its kinase activity. During mitosis the location of the protein changes, in metaphase the kinase localizes on the microtubules at the poles of the mitotic spindle whereas it is not present on astral microtubules. This localization persists until the segregation of the chromosomes is completed. The presence of the kinase on the spindle may reveal another yet unknown function. © 1999 Éditions scientifiques et médicales Elsevier SAS

Xenopus / centrosome / kinase / spindle

INTRODUCTION

The pEg2 cDNA was cloned by means of differential screening of a *Xenopus laevis* cDNA library based on the fact that its mRNA is adenylated in the eggs and becomes deadenylated soon after fertilisation (Paris and Philippe, 1990). pEg2 is a serine/threonine kinase that belongs to the new family of aurora/Ipl1-related kinases (Roghi *et al*, 1998). These kinases (see Giet and Prigent (1999) for review) have been named aurora/Ipl1-related kinases after the *Drosophila* aurora kinase, the first reported member of the family (Glover *et al*, 1995) and the *S cerevisiae* gene *IPL1* (Chan *et al*, 1993). Two members of this family in man have recently been found to be overexpressed in various human cancers, the human aurora/Ipl1-related kinase 1 (also known as aurora2/aik/ARK1/BTAK/STK15) and the human aurora/Ipl1-related kinase 2 (aurora1/ARK2/aik2) (Sen *et al*, 1997; Bischoff *et al*, 1998; Zhou *et al*, 1998; Tastuka *et al*, 1998). Overexpression of either the active form or the inactive form of one of the two kinases leads to polyploidy and in some cases to transformation, thereby indicating that a deregulation of one of the kinases is potentially oncogenic. The human aurora/Ipl1-related kinase 2 (HsAIRK2) localises in the midbody during telophase and seems to be involved in cytokinesis (Shindo *et al*, 1998; Terada *et al*, 1998; Tastuka *et al*, 1998). The human aurora/Ipl1-related kinase 1 (HsAIRK1) that localized in the centrosome is more likely to be the human orthologue of the *Xenopus* pEg2 kinase. Cultured cells in which HsAIRK1 has been overexpressed become aneuploid with multiple centrosomes, suggesting that

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the kinase is involved in the centrosome cell cycle control. Abnormal centrosome amplification has also been reported in cells lacking p53 (Fukasawa *et al*, 1996) a tumor suppressor protein involved in many aspects of cell cycle controls such as checkpoints in response to DNA damage and apoptosis (Morgan *et al*, 1997). Like other cell cycle regulators such as p34cdc2, cyclins B, the breast cancer-susceptibility BRCA or the subunits of the anaphase-promoting complex (Blair Zajdel and Blair, 1988; Bailly *et al*, 1989, 1992; Rattner *et al*, 1990; Tugendreich *et al*, 1995; Pockwinse *et al*, 1997; Hsu and White, 1998), p53 also associates with the centrosome indicating that this organelle must play a key role in the control of ploidy during cell division (Fukasawa *et al*, 1996).

In the case of the centrosome aurora/Ipl1-related kinases, it has been reported that the overexpression of active kinase yields aneuploid cells and a transformed phenotype which suggests a direct role of the kinase in cancer (Bischoff *et al*, 1998; Tatsuka *et al*, 1998; Zhou *et al*, 1998). Although the consequences of a deregulation of the aurora/Ipl1-related kinase activity in cells have been clearly established, the exact function of the kinase has not been clearly elucidated yet.

We concentrated our effort on the pEg2 kinase that can be referred as the *Xenopus laevis* centrosome aurora/Ipl1-related kinase 1 (XlAIRK1). In *Drosophila*, mutations of the aurora gene lead to the formation of monopolar mitotic spindles in which the centrosomes have been duplicated but not separated. This suggests that the aurora kinase is directly involved in centrosome separation (Glover *et al*, 1995). The inhibition of pEg2 activity during spindle assembly in *Xenopus* egg extracts also leads to monopolar spindles (Roghi *et al*, 1998). In this report we present evidence that like in aurora mutants spindle monopolarity is due to a centrosome separation defect.

If pEg2 is involved in the separation of the centrosomes, the kinase may ensure a second function once the spindle is assembled. Indeed from this time on the kinase pEg2 starts to localize at the pole of the spindle in metaphase. The same localization has been observed for several of these centrosome aurora/Ipl1-related kinases in various organisms (Gopalan *et al*, 1997; Kimura *et al*, 1997; Shindo *et al*, 1998). The protein kinase associates with the microtubules until the segregation of the chromosomes is completed (Roghi *et al*, 1998). In *Saccharomyces cerevisiae* the gene *IPL1* encoding for the only aurora-related kinase had first been reported to be involved in the control of ploidy and chromosome segregation (Chan and Botstein, 1993; Francisco *et al*, 1994). What is the function of pEg2 from metaphase to telophase? pEg2 has never been found to be associated either with the interphase microtubules or the astral microtubules of the mitotic spindle (Roghi *et al*, 1998). pEg2 can bind only to the microtubules at the pole of the spindle (Giet and Prigent, 1998; Roghi *et al*, 1998). However, *in vitro* pEg2 protein can bind directly to taxol-stabilized microtubules (Roghi *et al*, 1998). What is the relationship between pEg2, the centrosome and the microtubules? All these issues are presented and discussed in this paper.

MATERIALS AND METHODS

Spindle assembly assay

Demembranated sperm nuclei were prepared according to Lohka and Masui (1984). Fresh metaphase-arrested *Xenopus* egg extracts (CSF extract) were prepared as previously described (Roghi *et al*, 1998). 40 µg of rhodamine-labeled tubulin (TEBU) and about 10 000 demembranated sperm heads were added to 200 µL of freshly prepared CSF-arrested extracts (Masui and Markert, 1971) and incubated for 15 min at 22 °C. The exit from metaphase was triggered by addition of 0.4 mM calcium. After 45 min, when interphase nuclei were formed (Shamu and Murray, 1992), 20 µL of the extracts were driven into metaphase with the addition of 20 µL fresh CSF-arrested extract containing 4 µg of pEg2-K/R-(His)6 (Roghi *et al*, 1998). 1 µL of aliquots were fixed in a 2 µL Hoechst buffer (15 mM PIPES, pH 7.5, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 7.4% formaldehyde, 1 µg/mL bis-benzimide, 5 mM $MgCl_2$, 0.2 M sucrose) and mitotic spindles were observed by means of fluorescence microscopy with a Zeiss fluorescent microscope (Axiolab).

Indirect immunofluorescence microscopy

Xenopus XL2 cells were grown on glass coverslips and fixed through immersion in cold (-20 °C) methanol for 6 min, washed three times in a phosphate-buffered saline solution (PBS: 136 mM NaCl, 26 mM KCl, 2 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2), and blocked in PBS containing 3% BSA for 30 min. pEg2 was detected with the mouse monoclonal antibody 1C1 and γ-tubulin with a rabbit polyclonal antibody (Roghi *et al*, 1998). The antibodies were revealed by incubation with Texas red-conjugated goat anti-mouse IgG (dilution: 1/70) (Interchim) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution: 1/35) (Interchim), respectively. All antibody reagents were diluted in PBS containing 1% BSA and the incubations were performed at room temperature for 60 min. Cells were rinsed in PBS containing 1% BSA between each incubation and mounted in Mowiol containing antifade. Samples were observed using a Zeiss fluorescent microscope (Axiovert 35) and photographed using a Nikon 601 camera.

Immunoelectron microscopy

Immunoelectron microscopy was performed as previously described (Roghi *et al*, 1998). All treatments were at 25 °C. The cells were rinsed in PBS and fixed in cold

absolute methanol. After three washes in PBS, preparations were blocked for 30 min in PBS containing 3% BSA. Cells were then incubated with a primary anti-pEg2 1C1 monoclonal antibody (40 μg/mL), washed and incubated in secondary anti-mouse 15 nm gold-conjugated antibodies (dilution: 1/4) (Tebu, Le Perray-en-Yvelines, France). The antibody reagents were diluted in PBS containing 1% BSA and incubated with the antigen at room temperature for 60 min. Cells were then washed with PBS containing 1% BSA and fixed for 90 min in 0.1 M phosphate buffer, pH 7.2 (KH_2PO_4-Na_2HPO_4) containing 2.5% glutaraldehyde. After being rinsed with PBS, cells were post-fixed with 1% osmium tetraoxide, stained with uranium acetate, dehydrated and embedded in an Epon 812 mixture (Sigma Chemicals). Serial ultra-thin (70 nm) sections were obtained parallel to the substrate plane on the LKB-V ultramicrotome and mounted on single slot grids. The sections were examined using a Philips electron microscope operating at 80 kV and photographed.

Three-dimensional reconstitution

The 3D data sets were acquired from electron micrographs using a computer-linked Vidicon camera. The pictures were acquired as 8-bit 512 × 512 pixels frames with a resolution of 4.9 nm per pixel. Before digitizing, each picture was manually aligned with a previously acquired image using neighboring yolk granules as internal reference points (in our case, the reference points were chromosomes and centriolar cylinder where possible). After reliable image processing and binarisation, sequential images were stacked and surface rendered models were generated using a 'ray tracing' algorithm. The computer 3D models were viewed from various angles (in our case −10 and +10). Data acquisition, image processing, reconstitution and viewing of 3D-models were performed using a 'L-Vit' imaging system run on an IBM-compatible computer (Fais *et al*, 1996).

Immunoprecipitation

Xenopus egg extracts were prepared as described by Lohka and Maller (1988) and diluted 10 times in 20 mM Tris-HCl, pH 8, 150 mM NaCl, complemented with 0.5% NP40, 5 mM EDTA, 3 mM EGTA, 5 mM glycerophosphate, 0.5 mM sodium vanadate, 0.5 μg/mL leupeptin, pepstatin and chymostatin. Protein G Sepharose beads (Pharmacia CL6B) were conjugated with anti-pEg2 1C1 monoclonal antibodies through incubation at 4 °C for 1 h in the presence of an excess of antibodies diluted in PBS followed by extensive PBS washes. For immunoprecipitations, 10 μL of saturated beads were added to 50 μL of diluted egg extract and incubated at 4 °C for 1 h on a rotating wheel. After centrifugation, the beads were washed three times with 1 mL of dilution buffer followed by two washes with 1 mL of kinase buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT) and proceeded for kinase reaction.

Protein kinase assay

Assays were performed in 10 μL of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 1 μM [γ^{32}P] ATP 10 Ci/mmol (Amersham). The reactions were incubated at 37 °C for 15 min, stopped with the addition of 10 μL of 2 × Laemmli SDS-sample buffer and denatured at 90 °C for 10 min. The proteins were then separated by SDS-polyacrylamide gel electrophoresis, electro-transferred onto nitro-cellulose membranes and analysed by means of autoradiography. The presence of pEg2 in the immunoprecipitated proteins was monitored with Western blot.

Western blot analysis

The proteins were separated through SDS-polyacrylamide gels according to Laemmli (1970) and transferred onto nitro-cellulose membranes according to Towbin *et al* (1979). The membranes were blocked for 2 h at 4 °C in TBST containing 5% skimmed milk and incubated for 1 h at 20 °C TBST containing 2.5% skimmed milk and anti-pEg2 antibodies. Immuno-complexes were revealed with antibodies coupled with alkaline phosphatase (Sigma) using either NBT/ BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) as substrates or a chemoluminescence according to the manufacturer's instructions (NEN).

Activation of recombinant pEg2

The *Xenopus* egg extracts were prepared according to Newport and Maller (1985). The recombinant pEg2 proteins were purified as previously described (Roghi *et al*, 1998). 500 μg of purified recombinant protein were incubated for 1 h at 4 °C in 5 mL of *Xenopus* egg extract diluted 1/10 in TK20 (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 20 mM imidazol) containing a mixture of protease inhibitors (0.5 μg/mL leupeptine, pepstatine, tosyl-lysine-chloro-ketone (TLCK), 27 μg/mL aprotinine, 1 mM PMSF). The pEg2 protein was then re-purified through a Ni-NTA-agarose column (Qiagen).

RESULTS

pEg2 is an aurora/Ipl1-related kinase

pEg2 is a member of a new emerging family of mitotic kinases, the aurora/Ipl1-related kinases (AIRKs). At least one member of this family has been found in each of the eukaryote cells investigated so far, ranging from yeast to human (Francisco *et al*, 1995; Kimura *et al*, 1997; Sen *et al*, 1997). Higher eukaryote cells, such as human cells, contain several members of the family (three are present in human cells): a centrosome kinase (Kimura *et al*, 1997), a midbody kinase (Shindo *et al*, 1998; Prigent *et al*, 1998) and a kinase that localizes on centrosomes only in anaphase (Bernard *et al*, 1998; Tseng *et al*, 1998; Kimura *et al*, 1999). All these AIRKs share the same two domain structures, a C-terminal catalytic domain highly preserved throughout evolution and an N-terminal domain without any obvious common features that varies in size (fig 1A). The catalytic domain presents all

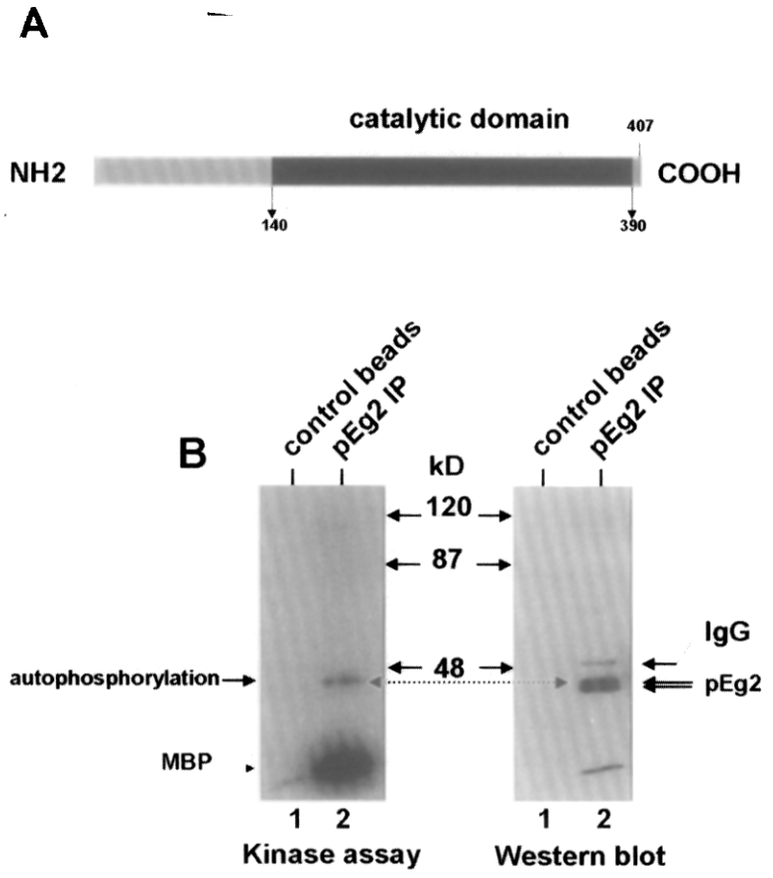

Fig 1. pEg2 is a 46 kDa protein kinase with a C-terminal catalytic domain that phosphorylates the myelin basic protein (MBP) *in vitro*. **A.** Schematic structure of pEg2. **B.** Immunoprecipitation of pEg2 from the *Xenopus* egg extract. The proteins attached to the protein G beads (lane 1) or to the protein G saturated with anti-pEg2 antibody (lane 2) were assayed for kinase activity using MBP as substrate (left panel) and controlled for the presence of pEg2 protein by Western blot (right panel).

the conserved serine/threonine kinase subdomains (Hanks *et al*, 1991).

We have developed a monoclonal antibody (1C1) against pEg2 (Roghi *et al*, 1998). This antibody immunoprecipitates a 46 kDa protein kinase in *Xenopus* egg extract that phosphorylates the myelin basic protein (MBP) *in vitro* (fig 1B, lane 2, left panel). Although pEg2 phosphorylates serine residues in the MBP, the sites are different from those phosphorylated by the MAP kinase (data not shown). pEg2 protein kinase also autophosphorylates (fig 1B, lane 2, left panel), this autophosphorylation provokes a mobility shift of the protein in SDS-polyacrylamide gel. pEg2 is immunodetected as a doublet in the immunoprecipitate, the upper band corresponds to the autophosphorylated protein (fig 1B, lane 2, right panel and left panel, dashed arrows). Within the doublet the proportion of the two forms seems to be 50% for each, indicating that the reaction responsible for the shift has reached an equilibrium.

A recombinant pEg2 protein kinase expressed in *E coli* is poorly active (Roghi *et al*, 1998), but is stimulated 2- to 4-fold after incubation in a *Xenopus* egg extract (fig 2A), indicating that a post-translational modification activates the kinase *in vivo*. When electrophoresed through a SDS-polyacrylamide gel, the mobility of the recombinant protein changed after incubation in the extract, and the activated kinase migrated faster (fig 2B). This modification is due to a phosphorylation of the kinase, because in the presence of [γ-^{32}P]ATP the inactive recombinant kinase became radioactive upon incubation in the extract (fig 2C). This kind of regulation has also been suggested for the human and mouse pEg2 orthologues specifically upon entering mitosis (Kimura *et al*, 1997; Gopalan *et al*, 1997). In the catalytic domain of serine/threonine kinases, the activation domain is located between subdomains VII and VIII. Like every member of aurora/Ipl1-related kinases cloned, pEg2 contains a conserved threonine that could be a target for an activating kinase.

pEg2 kinase activity is involved in centrosome separation

Nuclei were formed upon incubation of the CSF-extract with calcium and demembranated sperm nuclei. The addition of a CSF-extract to the nuclei drives the extract into mitosis and block its progression in metaphase (Shamu and Murray, 1992) (fig 3A). The addition of active recombinant pEg2

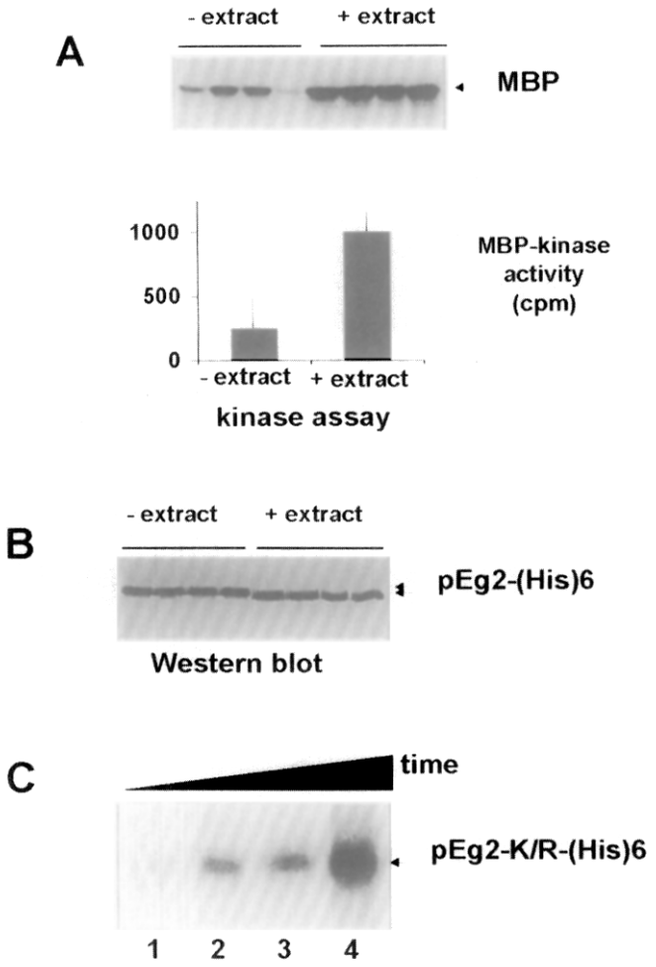

Fig 2. pEg2 kinase activity is stimulated by phosphorylation. **A.** Four separated experiments are presented, 500 µg recombinant pEg2-(His)6 were incubated in 5 mL of *Xenopus* egg extract diluted 1/10 (+ extract) and purified onto nickel column or kept on ice (– extract). The kinase activities of both proteins were compared using MBP as substrate. The samples were loaded onto a SDS-polyacrylamide gel. The activity of each protein was estimated by cut-off the MBP from the gel and counting the radioactivity incorporated. **B.** Immunodection of the recombinant proteins assayed for kinase activity after being incubated in *Xenopus* egg extract (right panel) or not (left panel). **C.** Time course of phosphorylation of inactive pEg2 in *Xenopus* cytoplasmic egg extract. 4 µg of pEg2-K/R-(His)6 were incubated with 4 µL of *Xenopus* egg extract diluted 1/75 in the presence of 2 µCi of [γ^{32}P]ATP (5000 Ci/mmol, Amersham) in 80 µL of reaction mixture. At various time during the incubation at 37 °C 20-µL aliquots were taken and analyzed by SDS-polyacrylamide electrophoresis. Lanes 1 to 4 are respectively 0, 15, 30 and 60 min of incubation at 37 °C.

protein to the fresh CSF extract had no detectable effect on the formation of mitotic bipolar spindles in metaphase. In contrast, the addition of an inactive recombinant pEg2 protein inhibits the formation of bipolar mitotic spindles; only monopolar spindles were formed (Roghi *et al*, 1998).

These data suggested that pEg2 kinase was required for the establishment of the bipolar mitotic spindles in *Xenopus* egg extracts. The inactive recombinant pEg2 protein was designed by exchanging the active lysine residue with an arginine residue in the catalytic domain. The protein acted as a dominant negative kinase, presumably by titrating partner(s) and/or substrate(s) of endogenous pEg2.

It was clear that pEg2 inhibition affected the spindle bipolarity. In most monopolar spindles, only one pole was observed (fig 3B–D) (Roghi *et al*, 1998). Here we show that in few of the monopolar spindles the two centrosomes have started to separate (fig 3E–G). The distance between those two centrosomes was far too small to allow the formation of bipolar spindles. These results indicate that the spindle monopolarity is a direct consequence of a centrosome separation defect. They also suggest that the activity of pEg2 may be directly involved in the separation of the centrosomes, a function reported for the *Drosophila* kinase aurora that might well be a pEg2 orthologue.

Localisation of pEg2

The cellular localization of the protein pEg2 was detected by means of indirect immunofluorescence in *Xenopus* XL2 cultured cells during the various stages of the cell cycle with the 1C1 monoclonal antibody. Cell cycle stages were identified with phase contrast (fig 4A, D, G, J), γ-tubulin staining indicated the centrosomes position (fig 4C, F, I, L). pEg2 was first detected at the end of S phase in the duplicated centrosomes (Roghi *et al*, 1998). The protein then remained associated with the centrosome throughout G2 and M (fig 4B, E, H, K). The association of pEg2 with the centrosome is weak because the staining was lost when cells were previously treated with detergents such as Triton X-100 (data not shown).

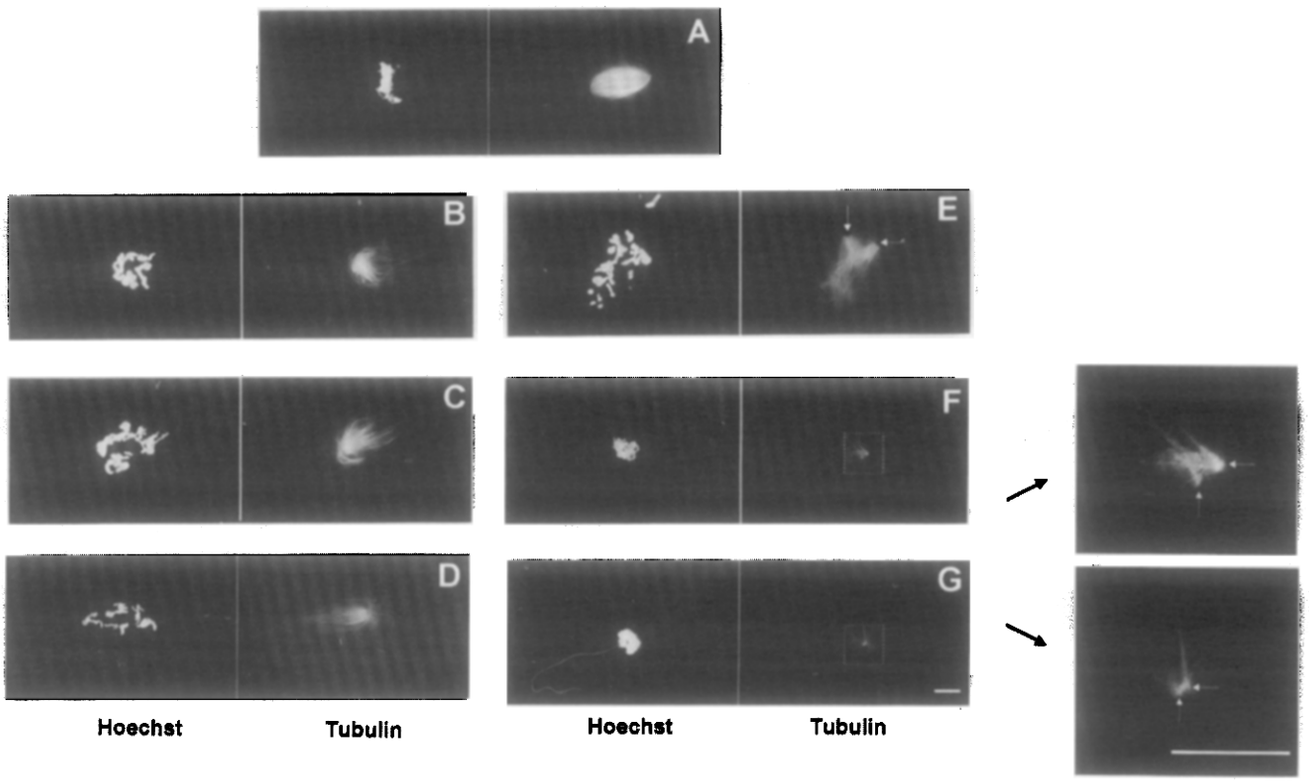

Fig 3. Inhibition of pEg2 activity leads to centrosome separation defect during *in vitro* spindle assembly in *Xenopus* egg extract. Reactions were started by adding fresh CSF extract (CSF1) to demembranated sperm heads. After addition of calcium nuclei formed within the first 45 min. Extracts were then driven into metaphase and arrested by the addition of new CSF-arrested extract (CSF2). Reactions were followed by fluorescence microscopy. Chromosomes were stained with DAPI and microtubules visualized by addition of rhodamine-labeled tubulin at the beginning of the reaction. **A.** Controlled reaction metaphase plate and mitotic spindle obtained 35 min after addition of CSF2. The same result was obtained when active pEg2 was previously added to CSF2 (Roghi *et al*, 1998). **B–G.** Monopolar spindles observed after addition of CSF2 containing pEg2-K/R-(His)6. **A–C.** Monopolar spindles in which the centrosomes are not resolved. **E–G.** Monopolar spindle in which the centrosomes are resolved (white arrows). Bar is 10 μm.

In mitosis pEg2 localization changes, when the microtubule spindle is assembled in metaphase, pEg2 localizes at the spindle pole (fig 3E). When pEg2 localizes in the centrosome the staining has a dot shape, whereas from metaphase to anaphase the staining shows a cup shape at both poles of the spindle as if the protein moved on the microtubules.

At the electron microscopy level, pEg2 is homogeneously found around the pericentriolar material in interphase (fig 5). In mitosis, the protein proportion increases at the spindle poles to decorate the spindle microtubules but not the astral microtubules (fig 6). Figure 6 is a pEg2 stereo-reconstruction of localization in the mitotic spindle; several serial electron microscopy pictures were used to build this image which was colored by computer. It is quite clear in this picture that pEg2 invades the metaphase spindle poles. Because pEg2 is not a motor protein, it may associate with a motor protein to move on the microtubule or it may directly bind to the microtubules, as gold particles are clearly found closely associated with the microtubules in anaphase, for instance (fig 7).

pEg2 and microtubules

A recombinant protein expressed and purified from *E coli* can directly bind *in vitro* to taxol-stabilized microtubules (Roghi *et al*, 1998). Furthermore, an inactive recombinant pEg2 protein also binds *in vitro* to taxol-stabilized microtubules and *in situ* to the mitotic spindle poles, indicating that the affinity for the microtubules is independent from the catalytic activity of the kinase (Giet *et al*, 1998). What is the relationship between pEg2 and the microtubules?

First, *in vivo* pEg2 is never found associated with interphase microtubules, but only with the mitotic microtubules and not the astral microtubules of the spindle (Roghi *et al*, 1998).

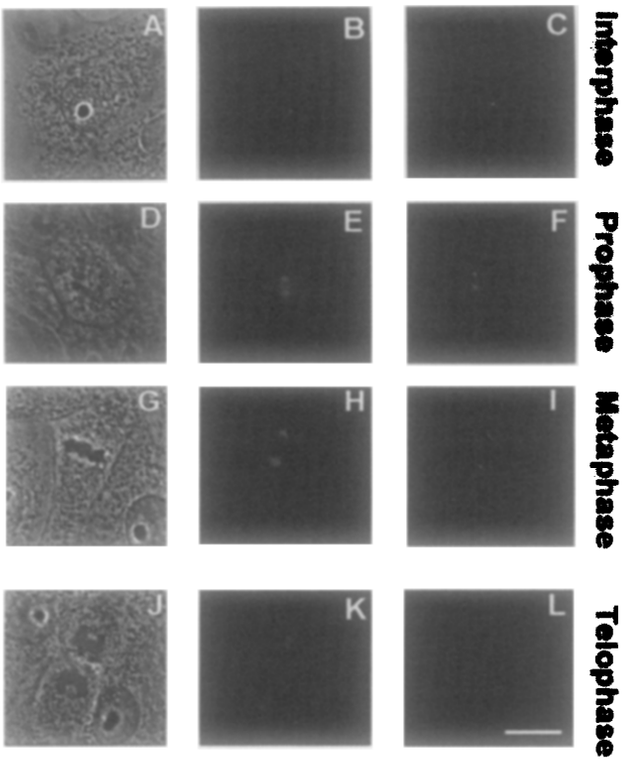

Fig 4. In *Xenopus* XL2 cells pEg2 localizes at the pole of the spindle in mitosis. pEg2 and gamma-tubulin were immunolocalized like previously described (Roghi *et al*, 1998). The stages of mitosis were determined using phase contrast microscopy (prophase (**A**); metaphase (**D**); anaphase (**G**); and telophase (**J**)). Cells were labeled for indirect immunofluorescence microscopy using anti-pEg2 antibody (**B, E, H, K**) and gamma-tubulin antibody (**C, F, I, L**). Bar is 10 μm.

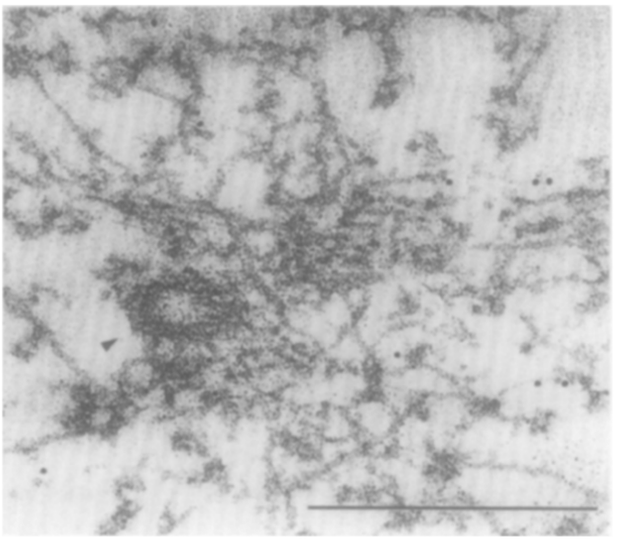

Fig 5. Ultrastructural localization of pEg2 around the centrosome in interphase cells. Immunoelectron microscopy was performed as previously described (Roghi *et al*, 1998). Anti-pEg2 antibody was revealed with a secondary antibody coupled to 15 nm gold particles.

In mitotic cells treated with the microtubule depolymerizing drug nocodazole, pEg2 still localizes in the centrosome indicating that the centrosome localization does not depend on the presence of the microtubules (Roghi *et al*, 1998). When mitotic cells are treated with taxol that stabilizes the microtubule, several microtubules asters appear in the cell, only two of them are γ-tubulin positive (centrosomes) and only those two are pEg2 positive (Roghi *et al*, 1998).

Although *in vitro* pEg2 can bind directly to microtubules, *in vivo* pEg2 may have a stronger affinity for a protein present only in mitosis at the spindle pole. The localization of pEg2 at the spindle poles may reveal the presence of one of its substrates.

DISCUSSION

The centrosome aurora/Ipl1-related kinase seems to control several aspects of the centrosome cell cycle. *Drosophila* aurora mutants in which the aurora gene encodes an inactive kinase show a centrosome separation defect (Glover *et al*, 1995). In *Xenopus* egg extracts, the inhibition of the pEg2 kinase affects the bipolarity of the mitotic spindle because centrosomes do not separate (Roghi *et al*, 1998; this article). Conversely, in the case of *Caenorhabditis elegans* disruption of the kinase AIR-1 affects neither the centrosome separation nor the spindle bipolarity but yields abnormal centrosomes instead (Schumacher *et al*, 1998). Overexpression of the kinase in human cells generates centrosome amplification (Zhou *et al*, 1998). These multiple effects observed after alteration of kinase activity may be the consequence of a deregulation of several different substrates.

One of the striking results observed is linked to the control of cell cycle progression. It is now established that the centrosome has its own cell cycle clock that can run quite independently of the nuclear cycle clock. The inhibition of DNA synthesis (*eg* with hydroxyurea or aphidicolin) blocks cell cycle progression in the S phase (D'anna *et al*, 1985). The cell cannot enter the M phase until DNA synthesis is completed (Saka *et al*, 1994; D'Urso *et al*, 1995). Under these conditions, the centrosome cycle is not affected. Centrosomes keep duplicating, leading to an amplification of centrosome pairs (Kuriyama *et al*, 1986; Balczon *et al*, 1995; Gorgidze and Vorobjev, 1995). A worthwhile result has been reported after overexpression of the centrosome aurora/Ipl1-related kinase in human cells. Not only

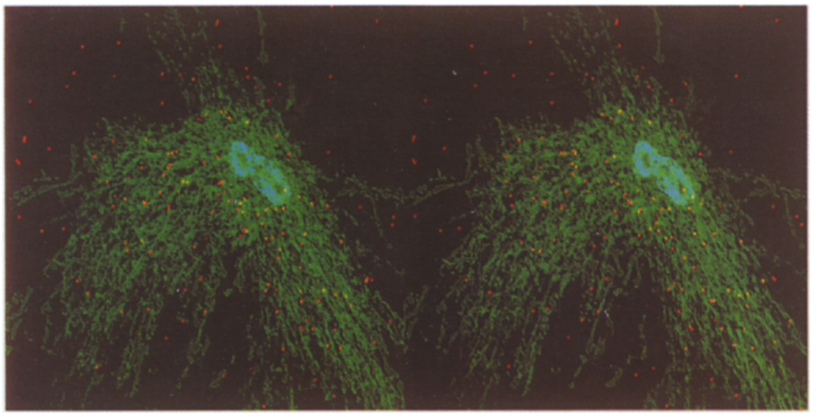

Fig 6. Three-dimensional reconstitution of a mitotic spindle pole. Microtubules are in green, centrosomes in blue and pEg2 in red. Bar is 1 μm.

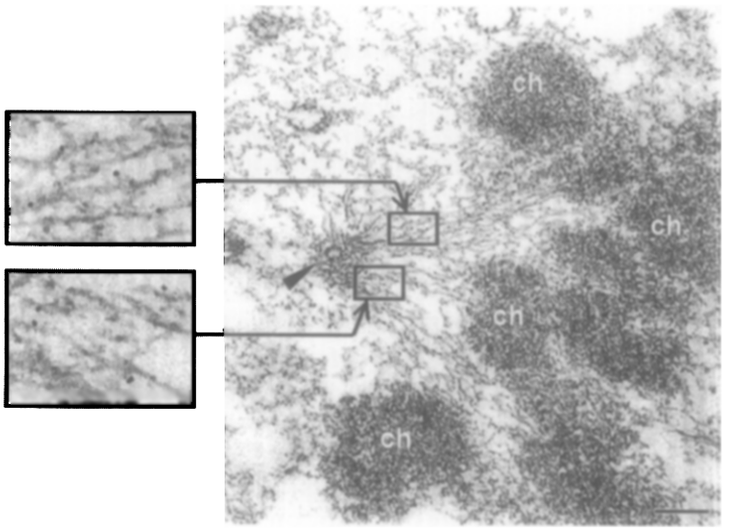

Fig 7. Ultrastructural localization of pEg2 on spindle microtubules during anaphase. Immunoelectron microscopy was performed as previously described (Roghi *et al*, 1998). Anti-pEg2 antibody was revealed with a secondary antibody coupled to 15 nm gold particles. Both enlargements clearly show that pEg2 is located on microtubules.

do the cells contain multiple centrosomes, but they become polyploid, thereby indicating that cell cycle progression is not affected even if the mitotic spindle cannot be assembled. Yet, eukaryotic cells possess a mitotic checkpoint that arrests cell cycle progression if all the kinetochores of the chromosomes have not captured the microtubules (Rieder *et al*, 1994). How can the cell exit from mitosis when a monopolar spindle is assembled instead of a bipolar spindle? One can postulate two hypotheses: 1) even with a monopolar spindle the kinetochores can capture microtubules, then the mitotic checkpoint remains silent and the cell exits from mitosis without completing chromosome segregation; and 2) because the spindle is monopolar, the kinetochores cannot capture microtubules. Under these conditions, the cell can exit from mitosis only by overcoming the mitotic checkpoint which tends to indicate that the centrosome aurora/Ipl1-related kinase is a checkpoint component.

The fact that pEg2 associates with the microtubules at the spindle poles is also intriguing. The kinase activities of pEg2 and some of its orthologues are believed to be involved in centrosome separation (Glover *et al*, 1995; Roghi *et al*, 1998). The localization of the protein in the centrosome is in agreement with such a function. Why does the protein also localize at the spindle poles from metaphase to anaphase? pEg2 could play an active role in the stabilization of the spindle poles like it has been shown for other spindle pole proteins. The microtubule spindles assembled in *Xenopus* egg extracts from which the protein NUMA has been previously depleted remain bipolar but do not possess distinct poles (Merdes *et al*, 1996). Although the bipolar spindle looks like a very stable structure when observed in metaphase for instance, its a very dynamic structure with high rate of microtubule polymerization/depolymerization. The stability of such a dynamic structure is achieved by the coordi-

nation of various microtubule-based motor protein activities. A model for the role of the different motor proteins has recently been proposed (Walczak *et al*, 1998). We are currently investigating the role of pEg2 in the stability of the spindle. Comprehension of the exact effect of pEg2 inhibition would necessarily give precious clues for the identification of its substrates.

pEg2 may also localize at the spindle poles for a reason independent of the dynamic of the spindle itself. Indeed, the ubiquitin degradation machinery for instance that controls the exit from mitosis is very closely related to the spindle microtubules (Kubiak *et al*, 1993; Wojcik *et al*, 1995). Many proteins degraded through this pathway localize in the mitotic spindle. This is the case of cyclin B during early syncytial divisions of *Drosophila* embryos, only a small proportion of cyclin B needs to be degraded to inactivate cdk1/cyclinB and trigger exit from mitosis. This population of cyclin B is locally degraded at the centrosome and at the chromosome kinetochore (Su *et al*, 1998). Although there is no direct evidence yet that the centrosome aurora/Ipl1-related kinases are degraded upon the exit from mitosis, the protein level is maximal in G2 and M phases of the cell cycle whereas it dramatically falls in G1 (Gopalan *et al*, 1997; Kimura *et al*, 1997). Also all aurora/Ipl1-related kinases, without any exception, contain in the C-terminal part of the protein a conserved amino acid sequence that may serve as a degradation box. The presence of pEg2 at the spindle poles may reflect its targeting for the ubiquitin degradation pathway.

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