The *Xenopus* protein kinase pEg2 associates with the centrosome in a cell cycle-dependent manner, binds to the spindle microtubules and is involved in bipolar mitotic spindle assembly

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SUMMARY

By differential screening of a *Xenopus laevis* egg cDNA library, we have isolated a 2,111 bp cDNA which corresponds to a maternal mRNA specifically deadenylated after fertilisation. This cDNA, called Eg2, encodes a 407 amino acid protein kinase. The pEg2 sequence shows significant identity with members of a new protein kinase sub-family which includes Aurora from *Drosophila* and Ipl1 (increase in ploidy-1) from budding yeast, enzymes involved in centrosome migration and chromosome segregation, respectively. A single 46 kDa polypeptide, which corresponds to the deduced molecular mass of pEg2, is immunodetected in *Xenopus* oocyte and egg extracts, as well as in lysates of *Xenopus* XL2 cultured cells. In XL2 cells, pEg2 is immunodetected only in S, G_2 and M phases of the cell cycle, where it always localises to the centrosomal region of the cell. In addition, pEg2 'invades' the microtubules at the poles of the mitotic spindle in metaphase and anaphase. Immunoelectron microscopy experiments show that pEg2 is located precisely around the pericentriolar material in prophase and on the spindle microtubules in anaphase. We also demonstrate that pEg2 binds directly to taxol stabilised microtubules in vitro. In addition, we show that the presence of microtubules during mitosis is not necessary for an association between pEg2 and the centrosome. Finally we show that a catalytically inactive pEg2 kinase stops the assembly of bipolar mitotic spindles in *Xenopus* egg extracts.

Key words: Kinase, Centrosome, Microtubule, *Xenopus*

INTRODUCTION

In higher eukaryotic cells microtubule dynamics are tightly regulated during the cell cycle (Belmont et al., 1990; McNally, 1996). The dynamics and functions of microtubules differ depending on cell type and which phase of the cell cycle the cell has reached (Sharp et al., 1995; Yu and Baas, 1994). In interphase, the microtubule network is involved in the maintenance of cell shape whilst also participating in cell motility and contributing to organelle transport (Sheetz, 1996; Fishkind et al., 1996; Kaech et al., 1996; Avila, 1992; Bloodgood, 1992). At the onset of mitosis the interphase microtubule network depolymerizes and a more dynamic, bipolar microtubule structure (the mitotic spindle) is formed which participates in the segregation of chromosomes.

Microtubule nucleation occurs at the centrosome which is the principal microtubule organising center (MTOC) in the cell (Pereira and Schiebel, 1997). The centrosome is a very complex structure, comprised of two perpendicularly oriented centrioles surrounded, in mitosis, by an electron dense filamentous matrix called the mitotic 'halo' or pericentriolar material (PCM). In some eukaryotic cells, the centrosome does not possess any centrioles (Brenner et al., 1977; Gueth-Hallonet et al., 1993) but it can still nucleate microtubules, suggesting: (i) that the nucleation of microtubules is independent of the presence of centrioles; and (ii) that the PCM contains all the factors required for mitotic microtubule nucleation (Moritz et al., 1995). For example, in *Xenopus* mitotic cytoplasm prepared from egg extracts, nucleation of bipolar mitotic spindles has been achieved in the absence of centrosomes and kinetochore DNA, demonstrating that the cytosol possesses all the factors necessary to nucleate the spindle microtubules (Heald et al., 1996). In interphase, the centrosome generally lies adjacent to the nucleus whereas, in mitosis, one centrosome is found at each pole of the mitotic spindle.

During the cell cycle, the centrosome and its microtubule nucleation activity undergo various changes (Kuriyama and

Borisy, 1981). In G_1, cells possess a single centrosome which nucleates the interphase microtubule array. Centrosome duplication occurs in late G_1 or in S phase independently of DNA replication (Kuriyama et al., 1986; Balczon et al., 1995; Gorgidze and Vorobjev, 1995). Once duplication has occurred, the new centrosomes migrate (between the end of G_2 and prometaphase) around the nucleus to lie on either side of this organelle by the beginning of metaphase. Upon entry into mitosis, specific interphase centrosomal structures such as pericentriolar satellites, free microtubule-nucleating focuses, primary cilia and striated rootlets disappear (Bornens, 1992). From their new positions both centrosomes nucleate microtubules, generating the bipolar mitotic spindle required for chromosome segregation (McIntosh, 1991). After cell division each daughter cell inherits a copy of the genome as well as one parental centrosome.

The regulation of the centrosome cycle during cell cycle progression (duplication, migration and MTOC activity) is far from being understood at the molecular level. The trigger for microtubule nucleation is still unknown, for instance. As for other cellular mechanisms regulated in a cell cycle-dependent manner, phosphorylation plays a central role (Karsenti et al., 1984; Nigg et al., 1996). The phosphorylation state of centrosomal proteins changes upon entry into and exit from mitosis (Vandré and Borisy, 1989; Centonze and Borisy, 1990; Vandré and Burry, 1992). In addition, phosphorylation by p34^{cdc2} controls the depolymerization of the microtubule network during the interphase-metaphase transition (Verde et al., 1990; Aizawa et al., 1991). Several protein kinases have been located in the centrosome, namely cyclic-AMP-dependent protein kinase type II (Nigg et al., 1985); p34^{cdc2} kinase (Bailly et al., 1989); Ca^{2+}/calmodulin-dependent protein kinase II (Ohta et al., 1990; Pietromonaco et al., 1995); casein kinase I (Brockman et al., 1992); polo-like kinase (plk1) (Golsteyn et al., 1995); LK6 kinase (Kidd and Raff, 1997); Aurora/Ipl1-related kinase (Aik: Aurora/Ipl1-related kinase) (Kimura et al., 1997); and Ipl1- and Aurora-related kinase 1 (IAK1) (Gopalan et al., 1997). Other proteins such as cyclin A and cyclin B (Maldonado-Codina and Glover, 1992), as well as the protein phosphatases X (Brewis et al., 1993) and 2A (Sontag et al., 1995) have also been immunolocalized to the centrosome.

In the present paper we report the discovery of a new protein kinase that associates with the centrosome in cultured cells and is expressed during *Xenopus laevis* early development. Between fertilization and the mid-blastula transition (MBT), the *Xenopus* embryo is engaged in a very intense cell division program with cells dividing approximately every 30 minutes (Newport and Kirschner, 1982). During this period, cell cycle progression is totally independent of RNA transcription. Control of post-fertilization cell proliferation relies on: (i) proteins already present in the egg; and (ii) newly synthesized proteins translated from maternal mRNAs that have been previously stockpiled in the egg (Newport and Kirschner, 1982). Differential screening of a *Xenopus* egg cDNA library has been undertaken in order to isolate mRNAs encoding for such proteins (Paris and Philippe, 1990). Nine clones were selected, based on the adenylation behavior of their corresponding mRNAs before and after fertilization (Paris and Philippe, 1990). They have been named Eg because their mRNAs are polyadenylated and translated in the eggs. Two of the Eg proteins have already been

characterized: Eg1 is the *Xenopus* p33^{cdk2} that controls the G_1/S transition in higher eukaryotes (Paris et al., 1991; Pagano et al., 1993); and p135^{Eg5} is a kinesin-related protein required for mitotic spindle assembly (Le Guellec et al., 1991; Sawin et al., 1992). Both proteins have been shown to be essential for cell cycle progression (Sawin et al., 1992; Paris et al., 1994). Here we report the cloning of a third member of the Eg family, pEg2, which has homology to a family of protein kinases. The protein encoded by Eg2 mRNA has been expressed in *E. coli* and purified as an active recombinant protein kinase. In addition we have found that in *Xenopus* cultured cells pEg2 associates with the centrosome in a cell cycle-dependent manner. We have also shown that pEg2 'invades' the microtubules at the poles of the spindle during mitosis. We have demonstrated that pEg2 binds directly to taxol stabilised microtubules in vitro and stays associated with the centrosome in cells with depolymerized microtubules. Using an inactive pEg2 kinase, designed to be a dominant negative, we found that bipolar spindles were not formed in *Xenopus* egg extracts in the presence of this protein. This suggests that pEg2 is involved in the correct formation of bipolar mitotic spindles.

MATERIALS AND METHODS

Cell culture and animals

Xenopus A6 cells (adult kidney) (a gift from Michel Kress, IRSC, Paris) and *Xenopus* XL2 cells (embryonic cell line) (Anizet et al., 1981) were grown at 25°C, without CO$_2$ in L15 Leibovitz medium (GibcoBRL) supplemented with 10% fetal calf serum (Bio Times) and antibiotic-antimycotic (Gibco BRL). Cells were cultured on round coverslips in 80 cm^2 plastic flasks or in 12-well plates (Corning Inc) for 48 hours. Conditions for nocodazole treatment were set up by incubating 48 hour cultured cells in medium containing 10 µg/ml nocodazole (Sigma Chemicals) for 2, 6, 9 and 12 hours. From pilot experiments we have chosen the 6 hour incubation as the time point after which all mitotic and more than 95% of interphase microtubules were depolymerized (data not shown). For taxol treatment, 48 hour cultured cells were incubated for 6 hours in medium containing 10 µg/ml taxol (Sigma Chemicals). Cells were then processed for immunofluorescence as described below. *Xenopus laevis* oocytes and eggs were obtained from laboratory-reared females. Eggs were artificially fertilised and embryos were staged according to the method of Nieuwkoop and Faber (1967). Germinal vesicles were prepared from stage VI oocytes as described by Dabauvalle et al. (1988).

cDNA cloning, sequencing and sequence analyses

Eg2 cDNA was isolated by differential screening of a *Xenopus* egg cDNA library as already described (Paris et al., 1988). Sequences were determined on both strands according to the method of Sanger et al. (1977). Searches in databases and sequence comparisons were performed with BLAST and FASTA programs (Pearson and Lipman, 1988).

Northern blot analysis

Total RNA was extracted from oocytes, eggs and embryos according to the method of Auffray and Rougeon (1980). Samples were fractionated by electrophoresis in 1% agarose gels containing 20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA and 6% formaldehyde, and transferred to nitrocellulose membrane (Amersham) according to the method of Alwine et al. (1977). The probe, corresponding to the first 1,178 nucleotides of the Eg2 cDNA, was ^{32}P-labelled with a random primer DNA labeling kit according to the manufacturer's instructions (Pharmacia). Membranes were

incubated with the probe for 4 hours at 42°C in 50% formamide, 1% SDS, 0.2% BSA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 10% dextran sulfate, 0.1% inorganic pyrophosphate, 1 M NaCl, 50 mM Tris-HCl, pH 7.5, 100 μg/ml denatured salmon sperm DNA, washed and autoradiographed.

Histidine fusion proteins

The coding sequence of Eg2 was engineered and amplified by polymerase chain reaction to be inserted in *Nhe*I/*Xho*I restriction sites of the His-tag expression vector pET21 (Novagen Inc.) using standard procedures (Sambrook et al., 1989). Oligonucleotides used as PCR primers contained a *Nhe*I restriction site (underlined) in the forward primer: 5′-CCCCCC<u>GCTAGC</u>GAGCGGGCTGTTAAGGAGAACC-AC-3′ and a *Xho*I site (underlined) in the reverse primer: 5′-CCCCCCC<u>CTCGAG</u>TTGGGCGCCTGGAAGGGGCTCGTC-3′. The resulting fusion protein has two extra amino acids (Arg-Ser) after the vector's methionine followed by pEg2 sequence and eight extra amino acids at the carboxyl end (Leu-Asp-His-His-His-His-His-His). A catalytically inactive protein kinase, pEg2-K/R-(His)6, was prepared by site-directed mutagenesis using the Transformer Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Clonetech). We used the mutagenesis primer 5′-GGATTTAAACAGGACT<u>C</u>TCAGCGCCAGG-3′ to change the lysine 169 to an arginine in pEg2-(His)6 protein sequence and the pET21 oligonucleotide 5′-GCGCAGCCAT<u>C</u>ACCCAGTCA-CGTAGCGATAGCGG-3′ (containing a mutated *Tth*111I site) as selection primer. The proteins were overexpressed in the *E. coli* strain BL21(DE3) (Novagen Inc.). Bacteria were grown at 37°C in LB medium containing 200 μg/ml ampicillin until OD_{600} reached 0.7. Overexpression was induced using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours at 25°C. pEg2-histidine-tagged protein was then purified on a nickel column (Ni-NTA-agarose) according to the manufacturer's instructions (Qiagen S.A.).

In vitro transcription and translation

The entire Eg2 cDNA was subcloned into pBluescript KS+ and the recombinant plasmid was linearized by *Sac*I. Eg2 mRNA was transcribed in vitro using the T7 RNA polymerase Riboprobe transcription system (Promega). For each in vitro translation reaction, 1 μg of Eg2 mRNA was translated at 30°C for 1 hour in 17.5 μl of rabbit reticulocyte lysate (Promega).

Western blot analysis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Proteins were electro-transferred to nitrocellulose membranes as described by Towbin et al. (1979). Membranes were blocked in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 5% skimmed milk for 2 hours at 4°C and incubated at 4°C for 1 hour with antibodies diluted in TBST containing 2.5% skimmed milk (anti-pEg2 1C1 monoclonal antibody was always used at a 1/1,000 dilution of the hybridoma culture medium and affinity purified anti-pEg2 polyclonal antibody was used at a 1/500 dilution). Immunocomplexes were revealed with an alkaline phosphatase-conjugated anti-mouse antibody or anti-rabbit antibody (Sigma Chemicals) using nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Sigma Chemicals) as substrates according to the manufacturer's instructions. Anti-p33[cdk2] polyclonal antibody (serum P5, a gift from Tim Hunt) was used as previously described by Chevalier et al. (1995). Anti-DNA ligase I polyclonal antibody was used as previously described by Aoufouchi et al. (1992). For western blot, anti-β-tubulin monoclonal antibody (Sigma Chemicals) was used according to the manufacturer's instructions.

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 MgCl2, 10 μM ATP containing 1 μCi [γ-[32P]ATP at 5,000 Ci/mmole and 100 ng of pEg2-(His)6 and/or 5 μg of MBP (myelin basic protein) as indicated. After 15 minutes incubation at 37°C, reactions were stopped by the addition of Laemmli sample buffer, and denatured by incubation at 90°C for 10 minutes. Reaction products were then analysed by western blot. Membranes were autoradiographed and eventually probed with antibodies as described.

Preparation of anti-pEg2 antibodies

For the rabbit polyclonal antibodies, a fusion protein containing the first 42 amino acids of the N-terminal domain of pEg2 was overexpressed in *E. coli*, purified and used for subcutaneous injections. The collected blood was centrifuged and specific anti-pEg2 IgGs were immunopurified from the serum using membrane-bound antigen as described (Harlow and Lane, 1988). For the monoclonal antibody, one mouse received several peritoneal injections of purified pEg2-(His)6 (75 μg each time). After a positive test bleed, the mouse received an intravenous injection of 75 μg pEg2-(His)6 and was sacrificed for hybridoma fusions. The screen for positive hybridomas and the selection of monoclonal antibodies were performed by ELISA using purified pEg2-(His)6. Positive responses were confirmed by western blot against pEg2-(His)6 and one monoclonal antibody named 1C1 was selected following confirmation of its ability to immunodetect pEg2 in *Xenopus* egg protein extract.

Microtubule co-pelleting assays

Polymerization of microtubules from purified tubulin was performed at 37°C for 30 minutes in BRB80 buffer (80 mM Pipes, 1 mM MgCl2, 1 mM EGTA, pH 6.8) containing 3 μM ATP, 3 μM GTP, 1 mM MgCl2, 2 μg pEg2-(His)6, 37.5 μg of purified tubulin and 25 μM taxol. Polymerization of microtubules from endogenous tubulin was performed in *Xenopus* egg cytosolic protein extracts, prepared from eggs arrested in metaphase II of meiosis due to the activity of the cytostatic factor (CSF) (Darr et al., 1991). CSF extracts were prepared according to the method of Morin et al. (1994) and were centrifuged at 70,000 rpm for 20 minutes at 4°C in a TL100A rotor using a Beckman TL100 ultracentrifuge. The pellet was discarded and the supernatant (metaphase cytosol) was transferred into new tubes (50 μl per reaction), kept on ice and supplemented with ATP regeneration mix (10 mM creatine phosphate, 80 μg/ml creatine kinase, 2 mM ATP and 1 mM MgCl2) and 10 μM taxol. Extracts were then incubated for 30 minutes at 23°C after adding further taxol to a final concentration of 25 μM (final reaction volume 100 μl). In all experiments, co-pelleting reactions were layered onto a 40% glycerol cushion in BRB80 containing 1 μM GTP and 10 μM taxol and centrifuged at 100,000 rpm for 30 minutes at room temperature in the TL100 rotor. The supernatant was conserved and the pellet was washed three times with BRB80. The supernatant (100 μl) was mixed with 25 μl of 5× Laemmli sample buffer (final volume: 125 μl) while the pellet was resuspended directly in 125 μl of 1× Laemmli sample buffer. Samples (2 μl of each fraction) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with various antibodies as described.

Spindle assembly assay

Demembranated sperm nuclei were prepared according to the method of Lohka and Masui (1984). Rhodamine-labelled tubulin was prepared according to the method of Hyman et al. (1991). Fresh metaphase-arrested *Xenopus* extracts (CSF extract) were prepared according to the method of Morin et al. (1994) from unfertilized egg (UFE) which are naturally arrested in metaphase II by cytostatic factor (CSF) (Masui and Markert, 1971). Rhodamine-labelled tubulin (40 μg) and demembranated sperm heads (10,000) were mixed with 200 μl of fresh CSF-arrested extracts. After 15 minutes incubation at room temperature (~22°C), calcium was added to a final concentration of 0.4 mM. Extracts were then incubated for a further 45 minutes until interphase nuclei had formed (Shamu and Murray, 1992). Extracts

containing interphase nuclei (20 μl) were then driven into metaphase by addition of fresh CSF-arrested extract (10 μl) containing either 100 ng of pEg2-(His)6 or 100 ng pEg2-K/R-(His)6. Samples (1 μl) were then fixed by addition of 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 spindle assembly was then assessed by fluorescence microscopy.

Indirect immunofluorescence microscopy

Xenopus laevis XL2 cells were grown on glass coverslips and fixed by immersion in cold (−20°C) methanol for 6 minutes. Following three washes in phosphate-buffered saline (PBS: 136 mM NaCl, 26 mM KCl, 2 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2), cells were blocked in PBS containing 3% BSA for 30 minutes and incubated with: mouse anti-β-tubulin monoclonal antibody (clone TUB2.1, Sigma chemicals, dilution: 1/200); mouse anti-pEg2 monoclonal antibody 1C1 (20 μg/ml) and/or rabbit anti-γ-tubulin polyclonal antibody (dilution: 1/100). The antibodies were revealed by sequential 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. For pEg2/γ-tubulin double staining experiments, we have taken great care not to use immunofluorescence stains that have overlapping emission spectra such as rhodamine and FITC (Carlsson and Ulfhake, 1995). We have used Texas Red for pEg2 staining and FITC for γ-tubulin staining. β-Tubulin staining was performed with Texas Red-conjugated goat anti-mouse IgG (dilution: 1/70). All antibody reagents were diluted in PBS containing 1% BSA and incubations were performed at room temperature for 60 minutes. Cells were rinsed in PBS containing 1% BSA between each incubation. Coverslips were rinsed in PBS and mounted in Mowiol containing antifade. Samples were observed using a Zeiss fluorescence microscope (AXIOVERT 35) and photographed using a Nikon 601 camera.

Immunoelectron microscopy

Cells on coverslips were briefly rinsed in PBS at 25°C and fixed for 6 minutes at −20°C in absolute methanol. After three washes (10 minutes each) in PBS at room temperature, preparations were blocked for 30 minutes at room temperature in PBS containing 3% BSA. Coverslips were then incubated with 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). All antibody reagents were diluted in PBS containing 1% BSA and all incubations were performed at room temperature for 60 minutes. After each incubation with antibody, cells were washed three times (10 minutes each) with PBS containing 1% BSA at room temperature. Cells were fixed for 90 minutes in 0.1 M phosphate buffer, pH 7.2 (KH_2PO_4-Na_2HPO_4), containing 2.5% glutaraldehyde. Cells were then rinsed in PBS, post-fixed with 1% osmium tetraoxide, stained with uranium acetate, dehydrated and embedded in Epon 812 mixture (Sigma Chemicals). Serial ultra-thin (70 nm) sections of cells were obtained parallel to the substrate plane on the LKB-V ultramicrotome and mounted on single slot grids. Sections were examined and photographed using a Philips electron microscope operating at 80 kV.

RESULTS

Eg2 cDNA encodes a putative protein kinase

The nucleotide sequence of Eg2 is shown in Fig. 1. Eg2 cDNA is 2,111 base pairs (bp) long and contains an open reading frame of 1,221 base pairs starting with an ATG at position 197 and ending with a stop codon at position 1,418. The presence of a stop codon at position 17 in frame and upstream of the ATG at position 197, strongly suggests that this is the real initiating codon. The 3′ untranslated region (3′UTR) is 691 bp long and is 61% AT rich with a polyadenylation signal AATAAA at position 2,077 and consensus sequences for targeting specific polyadenylation of mRNAs during *Xenopus* oocyte maturation (Fox et al., 1989; McGrew et al., 1989). A potential cytoplasmic polyadenylation element (U_{12}), active after fertilization, is also present in the 3′UTR (Simon et al., 1992). Several sequences involved in the specific degradation of Eg2 mRNA at the mid-blastula transition have also been identified in a segment of 17 nucleotides located at the proximal end of the 3′UTR (Bouvet et al., 1991).

Eg2 cDNA encodes a 407 amino acid protein, which includes all of the known characteristic sequences of serine/threonine kinases. The 12 conserved kinase sub-domains (Hanks et al., 1988) are present in a 250 amino acid catalytic domain located at the C-terminal end of the protein, between amino acids 140 and 390.

Protein kinases related to pEg2

A database search with the entire pEg2 protein sequence revealed significant identity to several protein kinases: Aik [human] (Kimura et al., 1997), STK-1 (serine/threonine kinase-1), IAK1, Ayk-1 (Aurora-family kinase 1) [*Mus musculus*] (Niwa et al., 1996; Gopolan et al., 1997; Yanai et al., 1997), Aurora [*Drosophila melanogaster*] (Glover et al., 1995) and Ipl1 [*Saccharomyces cerevisiae*] (Francisco et al., 1994). Percentages of identity were lower than 50%, with the exception of the human Aik protein kinase (Kimura et al., 1997) which was 62% identical to pEg2. Alignment studies employing only the catalytic domain sequence of pEg2, increased the identity to 80% between pEg2 and Aik, 75% for both STK-1 and IAK1, 73% for Ayk-1, 64% for Aurora and 50% for Ipl1. In this new protein kinase subfamily, only two members have been functionally characterised so far. Aurora and Ipl1 are involved in centrosome migration and chromosome segregation, respectively (Glover et al., 1995; Francisco et al., 1994).

All these kinases, including pEg2, possess a long extension of the amino-terminal domain (from 81 to 163 amino acids) and a very short C-terminal domain (maximum 17 amino acids). Sequences located outside the pEg2 catalytic domain (139 amino acids at the N terminus and 17 amino acids at the C-terminus) do not show any significant similarities with known proteins in databases.

Expression of Eg2 mRNA

A fragment corresponding to the first 1,178 nucleotides of the Eg2 cDNA was used as a probe for northern blot analysis of Eg2 mRNA expression during *Xenopus* oogenesis and early development. In agreement with the size of the cDNA, a single approximately 2.1 kb mRNA was detected in oocytes, unfertilised eggs and embryos (Fig. 2). We analysed Eg2 mRNA expression during oogenesis (Fig. 2, lanes 1 to 6) and observed a decrease in the signal when an equal amount of total RNA from different stages was loaded on the gel. The reduction in the signal intensity was proportional to the increase in total RNA, in particular ribosomal RNAs, which have previously been described during oogenesis (Pierandrei-Amaldi et al., 1982). After fertilisation, Eg2 mRNA was only detected before mid-blastula transition (Fig. 2, lane 9). Absence of a post-MBT signal (Fig. 2, lane 10) is the result of

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cggagaaggaaggaagtaagtggctgagctgctggggggggaagacagaggtgcgttccagtcgtgtgggaag    72
agggagcctcaggttttttgcgtctgcagctaaccttgccttgcctcgcctcgcctcgaacctaacta       144
tctgacagttgctacagactcggcctgcacatttagctgccaggcattcataATG GAG CGG GCT GTT     211
                                                      M   E   R   A   V    5
AAG GAG AAC CAC AAG CCT TCT AAT GTG AAG ATT TTT CAC CCC ATG ACT GAG GGA     265
 K   E   N   H   K   P   S   N   V   K   I   F   H   P   M   T   E   G      23
GCT AAA CGT ATC CCA GTT AAC CAG CCG CAG AGC ACT CAG TTT AGG CCT CCT GGG     319
 A   K   R   I   P   V   N   Q   P   Q   S   T   Q   F   R   P   P   G      41
ACT GCA GTC TCT GCC CAG AGG ATA TTG GGT CCC TCT AAT GTG CCG CAG CGA GTG     373
 T   A   V   S   A   Q   R   I   L   G   P   S   N   V   P   Q   R   V      59
TTG GCT CAG GCT CAG AAA CCT ATT CTG TCC AGT CAG AAG CCA ACG ACT CAG ATC     427
 L   A   Q   A   Q   K   P   I   L   S   S   Q   K   P   T   T   Q   I      77
CCC CTG CGG CCA GCA ACT CAA GGG CAT CAG TCC AGC AAA CCA CAG GGA CCA AAT     481
 P   L   R   P   A   T   Q   G   H   Q   S   S   K   P   Q   G   P   N      95
GAG AAT CGC AAT CCA CAG CAG ACA AGC CAT TCC TCC ACT CCA AAT GTG GAA AAG     535
 E   N   R   N   P   Q   Q   T   S   H   S   S   T   P   N   V   E   K     113
AAA GGA AGC ACC GAT CAA GGA AAA ACT TCA GCT GTT CCC AAA GAG GAG GGC AAA     589
 K   G   S   T   D   Q   G   K   T   S   A   V   P   K   E   E   G   K     131
AAG AAA CAA TGG TGC CTG GAA GAC TTT GAA ATA GGG CGG CCC CTG GGC AAA GGG     643
 K   K   Q   W   C   L   E   D   F   E   I   G   R   P   L   G   K   G     149
AAG TTT GGA AAT GTT TAT CTG GCA CGC GAG CGG GAG AGT AAA TTC ATC CTG GCG     697
 K   F   G   N   V   Y   L   A   R   E   R   E   S   K   F   I   L   A     167
CTG AAA GTC CTG TTT AAA TCC CAG CTA GAG AAG GCG GGC GTA GAG CAC CAG CTA     751
 L   K   V   L   F   K   S   Q   L   E   K   A   G   V   E   H   Q   L     185
CGG CGG GAG GTG GAA ATT CAG TCC CAT CTC AGG CAC CCC AAT ATT CTC CGG CTC     805
 R   R   E   V   E   I   Q   S   H   L   R   H   P   N   I   L   R   L     203
TAT GGC TAT TTC CAC GAT GCT TCC AGA GTC TAC CTA ATC CTG GAT TAT GCC CCT     859
 Y   G   Y   F   H   D   A   S   R   V   Y   L   I   L   D   Y   A   P     221
GGT GGG GAG TTG TTC AGA GAG CTG CAG AAA TGC ACA AGA TTT GAT GAT CAG AGA     913
 G   G   E   L   F   R   E   L   Q   K   C   T   R   F   D   D   Q   R     239
AGT GCC ATG TAC ATC AAA CAG CTG GCA GAA GCC CTC CTG TAC TGT CAC TCC AAG     967
 S   A   M   Y   I   K   Q   L   A   E   A   L   L   Y   C   H   S   K     257
AAG GTG ATA CAC AGG GAC ATC AAG CCA GAG AAC TTG CTG CTT GGC TCC AAT GGA    1021
 K   V   I   H   R   D   I   K   P   E   N   L   L   L   G   S   N   G     275
GAA CTG AAG ATC GCC GAC TTT GGC TGG TCT GTG CAT GCT CCA TCC TCC AGG AGG    1075
 E   L   K   I   A   D   F   G   W   S   V   H   A   P   S   S   R   R     293
ACC ACT CTG TGT GGA ACA CTG GAC TAT CTA CCT CCT GAG ATG ATC GAG GGC AGA    1129
 T   T   L   C   G   T   L   D   Y   L   P   P   E   M   I   E   G   R     311
ATG CAT GAT GAG ACG GTG GAC TTG TGG AGT CTG GGA GTT CTC TGT TAC GAA TTC    1183
 M   H   D   E   T   V   D   L   W   S   L   G   V   L   C   Y   E   F     329
CTC GTA GGG AAA CCT CCG TTT GAG ACT GAC ACC CAC CAG GAA ACC TAC AGA CGT    1237
 L   V   G   K   P   P   F   E   T   D   T   H   Q   E   T   Y   R   R     347
ATT TCA AAG GTT GAA TTT CAA TAT CCG CCA TAT GTA TCG GAA GAG GCA AGA GAT    1291
 I   S   K   V   E   F   Q   Y   P   P   Y   V   S   E   E   A   R   D     365
CTT GTC TCA AAG CTG TTA AAG CAC AAC CCA AAC CAC AGG CTG CCA CTG AAA GGG    1345
 L   V   S   K   L   L   K   H   N   P   N   H   R   L   P   L   K   G     383
GTT CTC GAA CAT CCA TGG ATC ATA AAA AAT TCT CAA CTC AAG AAG AAA GAC GAG    1399
 V   L   E   H   P   W   I   I   K   N   S   Q   L   K   K   K   D   E     401
CCC CTT CCA GGC GCC CAA TAG gaaatctcctgaggctccgactctcttcccaaatgtgagaaggc   1464
 P   L   P   G   A   Q   *                                                407
ataaccgtttgcacctgctagataacgcctcttattttttaattgggaggtggctaatccttccaaaagtgag  1536
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tttttttttttttttcttcttctgtttaaatgttttaaaataaagctttgattaaaaagaaaaaaaaaaaaa  2111
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Fig. 1. Features of the nucleotide sequence and predicted amino acid sequence of pEg2. Nucleotides sequence of Eg2 cDNA and deduced amino acid sequence of the pEg2 protein. Lower case characters in the cDNA represent non-coding sequence. Sequences underlined in the 3′UTR are: the potential polyadenylation signal (-AATAAA-); a specific post-MBT degradation element (-TGTCCTTTTATATGTAA-); two consensus sequences for polyadenylation during oocyte maturation (-TTTTAT- and -TTTTTAA-); and a potential cytoplasmic polyadenylation element (-TTTTTTTTTTTT-). These sequence data are available from GenBank under accession number Z17207.

Eg2 mRNA degradation due to its deadenylated state (Duval et al., 1990). This particular behaviour is characteristic of all the Eg mRNAs such as Eg1 (Paris et al., 1988) and Eg5 (Le Guellec et al., 1991).

The distribution of Eg2 mRNA was also analysed in different *Xenopus* adult tissues by northern blot. High level expression was only detected in ovary and testis (data not shown) in accordance with Eg1 and Eg5 mRNAs (Paris et al., 1990; Le Guellec et al., 1991). These data suggest that Eg2 mRNA may be expressed preferentially in dividing cells and therefore absence of the mRNA from most of the adult tissues analysed probably reflects the low level of cell division in these tissues. An Eg2 transcript (~2.1 kb) was also detected in RNA prepared from *Xenopus* XL2 and A6 cultured cells (data not shown).

Recombinant protein pEg2-(His)6 phosphorylates myelin basic protein in vitro

In order to study the kinase activity of pEg2, we subcloned the coding sequence of its cDNA into the pET21 bacterial expression vector to produce a histidine-tagged fusion protein (Studier et al., 1990). The soluble recombinant protein, overexpressed in the *E. coli* strain BL21(DE3), was affinity-purified using a nickel column and analysed on an SDS-polyacrylamide gel. The purified protein appeared as a triplet with an apparent molecular mass of 50 kDa for the top band (Fig. 3A, lane 4). Purification of an engineered pEg2-(His)6 mutant protein (^{169}K→R) which lacks kinase activity showed only the lowest band indicating that active pEg2-(His)6 has autophosphorylation activity in *E. coli* (see Fig. 11A, lane 2).
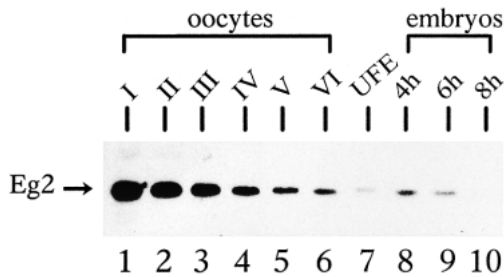
Fig. 2. Expression of Eg2 mRNA in *Xenopus* during oogenesis and early development. Total RNA (20 µg) from different stages of oogenesis and early development was separated on a formaldehyde-agarose gel and transferred to nitrocellulose membrane. The blot was hybridised with a probe prepared from the first 1,178 bp of the Eg2 cDNA.

Various commercially available potential kinase substrates, such as histone H1, alpha and beta caseins, and myelin basic protein (MBP), were used to investigate pEg2-(His)6 kinase activity in vitro. We found that pEg2-(His)6 readily phosphorylates MBP (Fig. 3B, lane 2). Histone H1 and beta casein were found to be poor substrates for the pEg2-(His)6 kinase, and no detectable activity was observed when using alpha casein (data not shown). We also found that the recombinant protein kinase autophosphorylates (Fig. 3B, lane 3) on a serine residue (data not shown).

Xenopus pEg2 is a 46 kDa cytoplasmic protein

In order to analyse the subcellular localisation of pEg2, we raised polyclonal and monoclonal antisera against the NH2-terminal domain and the entire protein, respectively. In western blot analyses, affinity-purified anti-pEg2 IgG detected a protein with an apparent molecular mass of 46 kDa in *Xenopus* unfertilised egg (UFE) extracts (Fig. 4A, lane 1). A pEg2 protein, translated in vitro in a rabbit reticulocyte lysate was also recognised by affinity-purified anti-pEg2 IgG (Fig. 4A, lane 2). A single band, with an apparent molecular mass of 46 kDa, was also recognized by anti-pEg2 monoclonal 1C1 in western blot analysis of protein extracts prepared from *Xenopus* UFE (Fig. 4B, lane 1), XL2 cells (Fig. 4B, lane 2), and A6 cultured cells (data not shown).

The localisation of pEg2 was first analysed in *Xenopus* stage VI oocytes. Germinal vesicles (nuclei) were manually isolated from the remains of the cell (cytoplasm) and each protein fraction was analysed by western blot, using the affinity-purified anti-Eg2 IgG. pEg2 protein was detected only in the cytoplasmic fraction (Fig. 4C, upper panel, lane 2) suggesting that the localisation of pEg2 is restricted to the cytoplasm of stage VI oocytes. In contrast, DNA ligase I was found only in isolated germinal vesicles (Fig. 4C, lower panel, lane 4) as expected (Aoufouchi et al., 1992).

### pEg2 associates with the centrosome in *Xenopus* XL2 cultured cells

Using the 1C1 monoclonal antibody, we examined the subcellular localisation of pEg2 by indirect immnnufluorescence microscopy of *Xenopus* XL2 cultured cells. Cell cycle stages were identified by phase contrast (Fig. 5A,E,I and M). The cells were double-stained with a rabbit anti-γ-tubulin polyclonal antibody (Fig. 5B,F,J and N) as a centrosomal
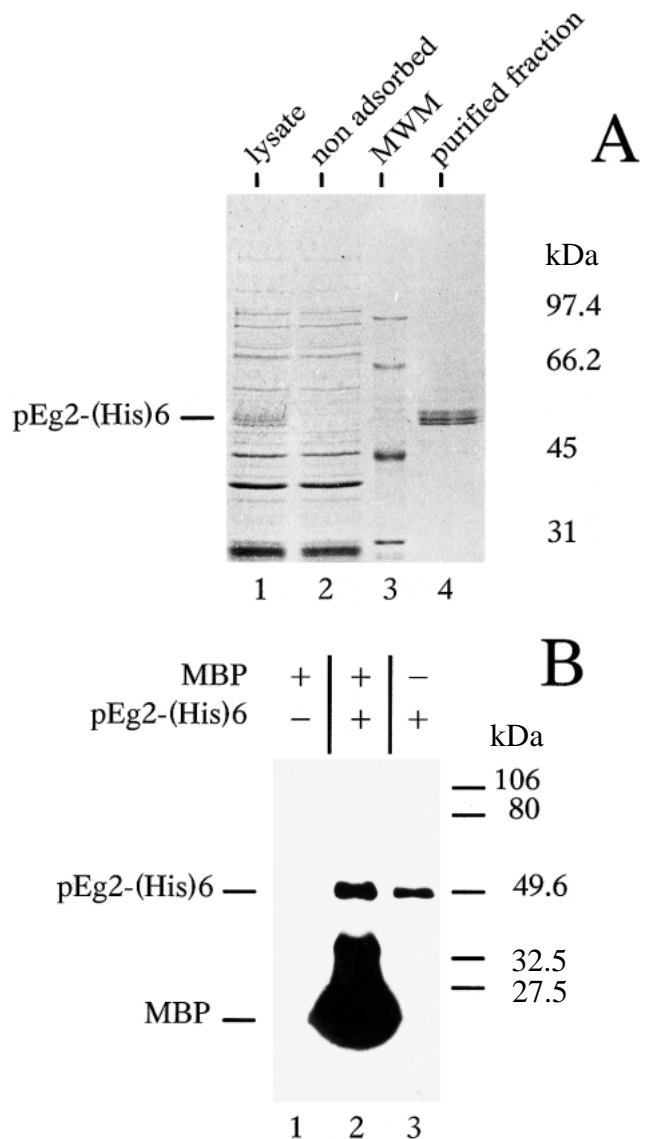
Fig. 3. Purification of an active recombinant protein kinase pEg2-(His)6. (A) Purification of pEg2-(His)6 by affinity chromatography on a nickel column. Lane 1, protein extract from *E. coli* overexpressing pEg2-(His)6; lane 2, flow through; lane 3, molecular mass markers (MWM); lane 4, purified pEg2-(His)6 eluted from the column using 250 mM imidazole. Proteins were fractionated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. (B) Assays for protein kinase activity of purified pEg2-(His)6. Phosphorylation reactions were performed at 37°C in kinase buffer (see Materials and Methods) containing 10 µCi of [γ-^{32}P]ATP (10 Ci/mmole), with the following additions: lane 1, 5 µg of MBP; lane 2, 5 µg of MBP and 0.1 µg of pEg2-(His)6; lane 3, 0.1 µg of pEg2-(His)6.

marker (Stearns et al., 1991) and with the mouse anti-pEg2 1C1 monoclonal antibody (Fig. 5C,G,K and O). We were unable to localise pEg2 in cell in late G1/early S phase (Fig. 5C) probably reflecting a low abundance of the protein or its cytoplasmic dispersion at these stages of the cell cycle. The protein was first detected at the end of S phase (Fig. 5G) and persisted until the end of mitosis (Fig. 5K and O and Fig. 6B,D,F and H).

Fig. 4. Identification of pEg2 as a cytosolic 46 kDa protein. (A) In vitro translation of Eg2 mRNA in reticulocyte lysate. Lane 1, control protein extract from one UFE; lane 2, translated Eg2 mRNA; lane 3, control lysate without added mRNA. Proteins were fractionated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and probed with affinity-purified anti-pEg2 polyclonal antibodies. (B) Immunodetection of pEg2 in XL2 cell lysate obtained by boiling directly the cells in Laemmli sample buffer) versus UFE extract obtained by boiling directly the UFE in Laemmli sample buffer. Protein extract equivalent to 0.1 *Xenopus* egg (lanes 2 and 4) and from 2×10⁵ XL2 cells (lanes 1 and 3) were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with (lanes 1 and 2) or without (lanes 3 and 4) anti-pEg2 monoclonal 1C1 antibody. (C) Sub-cellular localisation of pEg2 in *Xenopus* stage VI oocyte. Germinal vesicles were manually isolated from the remain of the cell. Proteins from one full stage VI oocyte (lane 1), one cytoplasm (Cy) (lane 2), one (lane 3) and 10 germinal vesicles (Gv) (lane 4) were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with affinity-purified anti-pEg2 polyclonal antibodies (upper panel) and anti-DNA ligase I rabbit polyclonal antibody (lower panel).
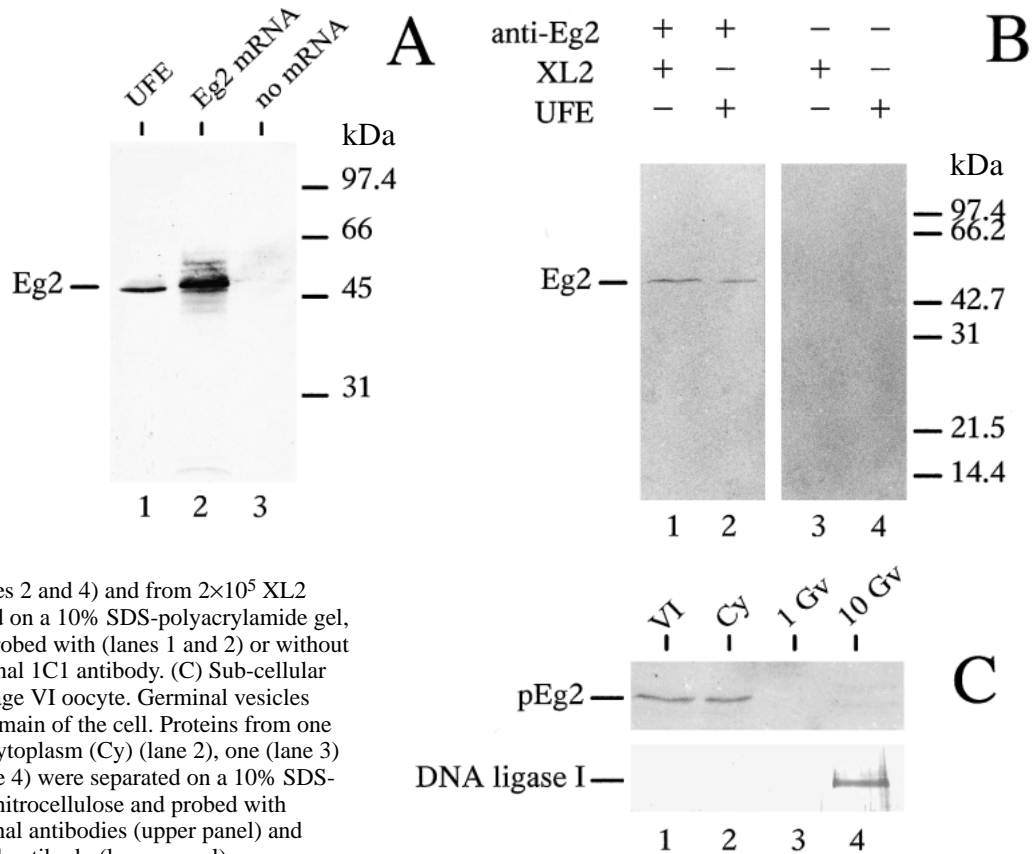
In localisation experiments, we found that the patterns of pEg2 and γ-tubulin fluorescence overlapped completely at the centrosome, with the pEg2 staining extending further along the spindle microtubules (Fig. 5D,H,L and P and Fig. 6B,D,F and H). We found no evidence of pEg2 elsewhere in the cytoplasm. In Fig. 5G, the anti-pEg2 1C1 monoclonal antibody stained two discrete foci, which are the two duplicated centrosomes. pEg2 was found to remain on the centrosomes during their migration around the nucleus (data not shown) and throughout mitosis (Fig. 5K and O and Fig. 6B,D,F and H). We have also noticed a strong competition between antibodies in our double staining (pEg2/γ-tubulin) experiments, which is likely to reflect a steric hindrance between the two antibodies and suggesting that both proteins may lie very close to one another.

During cell division, we observed a significant modification of the shape of pEg2 staining. In prophase, pEg2 staining appeared as a dot located at the centrosomal region of the cell (Fig. 6B). An enlargement of the picture revealed the presence of a small area lacking fluorescence at the center of the dot suggesting that pEg2 might be located around the centrosome and away from the centrioles (data not shown). In prometaphase, the shape of pEg2 staining was observed to change to resemble a cup-shaped staining at both poles of the spindle (Fig. 6D). The size of this cup-shaped staining increased, reaching its maximum in anaphase (Fig. 6F). In telophase, we observed again only the dot-shaped staining, and pEg2 was found to be restricted once more to a strict centrosomal location (Fig. 6H). Similar results were obtained with *Xenopus* A6 cultured cells (data not shown). Interestingly,

pEg2 was never seen on the astral microtubules. We have also noticed that the intensity of the pEg2 signal at the centrosome was stronger in mitosis than in interphase (Fig. 5K and O) suggesting: (i) a recruitment of pEg2 from a cytosolic pool during mitosis; or (ii) a modification of the intrinsic immunoreactivity of the protein; or (iii) an increase in Eg2 mRNA translation coupled with a recruitment to the centrosome.

In anaphase pEg2 co-localizes with microtubules of the mitotic spindle

In order to localise pEg2 precisely in the centrosomal region we performed immunoelectron microscopy experiments. At this level of resolution pEg2, revealed using 15 nm gold particles, was always found around the centrosome in interphase cells. Interestingly, pEg2 was never detected inside or on the centrioles nor associated with the pericentriolar satellites (data not shown). In mitosis, the centrosome has been characterised to consist of two domains; the two centrioles, which are surrounded by the pericentriolar material (PCM) (Bornens, 1992). Between prophase and anaphase, pEg2 protein was mainly found at the periphery of the PCM (Fig. 7A,B and C). However, this distribution appeared to change in metaphase with the presence of gold particles at the pole of the mitotic spindle (Fig. 7B). In anaphase, pEg2 is clearly co-localised with the microtubules near the poles (Fig. 7C).

Our observations clearly show that pEg2 is not associated with the centrioles nor trapped within the PCM, but sits around the outside of PCM during mitosis. They also confirm our

immunofluorescence data and provide a better view of the relocalisation of a fraction of pEg2 protein during mitosis.

pEg2 binds directly to microtubules

To determine whether or not pEg2 binds directly to microtubules, we performed microtubule-co-pelleting assays. Microtubules were polymerised in *Xenopus* metaphase cytosol in the presence of taxol before being pelleted. Supernatant and pellet were then tested, by western blotting, for the presence of pEg2. As expected, β-tubulin was only found in the microtubule pellet (Fig. 8A, upper panel, lane 1) whereas

p33[cdk2], which does not bind to microtubules, was only detected in the supernatant (Fig. 8A, middle panel, lane 2). Only a small fraction of pEg2 was able to pellet with the microtubule, leaving a large quantity of protein in the soluble fraction (Fig. 8A, lower panel, lane 1). This result suggests either that: (i) an insoluble fraction of pEg2 pellets in a microtubule-independent process; (ii) pEg2 binds poorly to microtubules; or (iii) pEg2 can bind to microtubules but the amount of microtubules polymerised in the metaphase cytosol is a limiting factor. In order to test this last hypothesis, we polymerised microtubules in metaphase cytosol in the presence
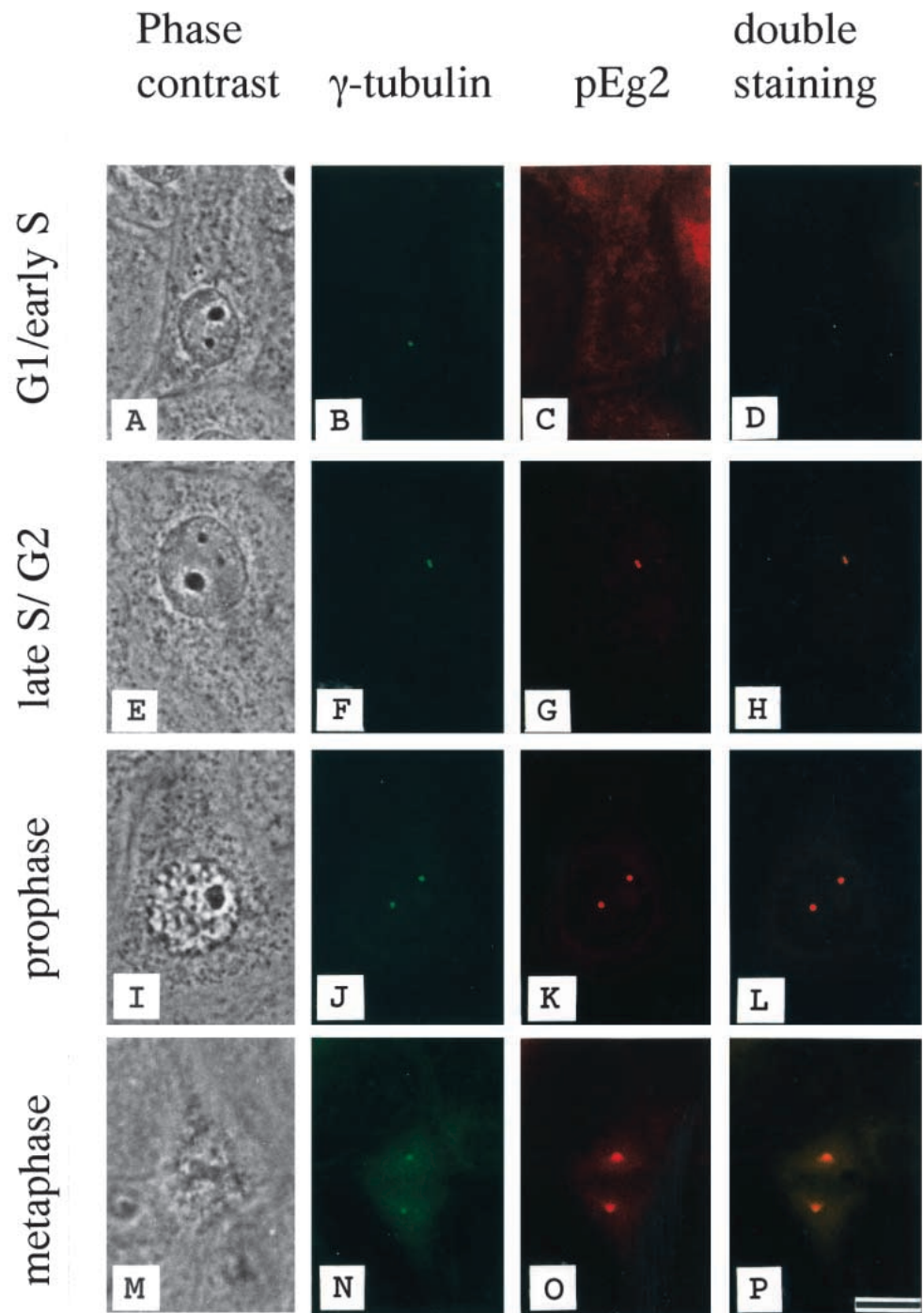
Fig. 5. Immuno-localisation of pEg2 during the cell cycle. The monoclonal anti-pEg2 antibody (1C1) was used to immunolocalise pEg2 in *Xenopus* XL2 cells. Cells were grown and fixed as described in Materials and Methods and labeled for indirect immuofluorescence microscopy with various antibodies: anti-γ-tubulin rabbit polyclonal antibody (B,F,J,N), anti-pEg2 1C1 monoclonal antibody (C,G,K,O), anti-pEg2 1C1 monoclonal and anti-γ-tubulin rabbit polyclonal antibodies (D,H,L,P). In several places, co-distribution of red fluorescence from pEg2 and green fluorescence from γ-tubulin labelling was significant enough to produce a yellow signal, indicating an overlap in the distribution of the two proteins. Cells were also observed using phase contrast microscopy (A,E,I,M) to determine the stage of the cell cycle. The cell in A to D is in G_1/early S phase, E to H is in late S phase/G_2, I to L is in prophase and M to P is in metaphase. Bar, 10 μm.
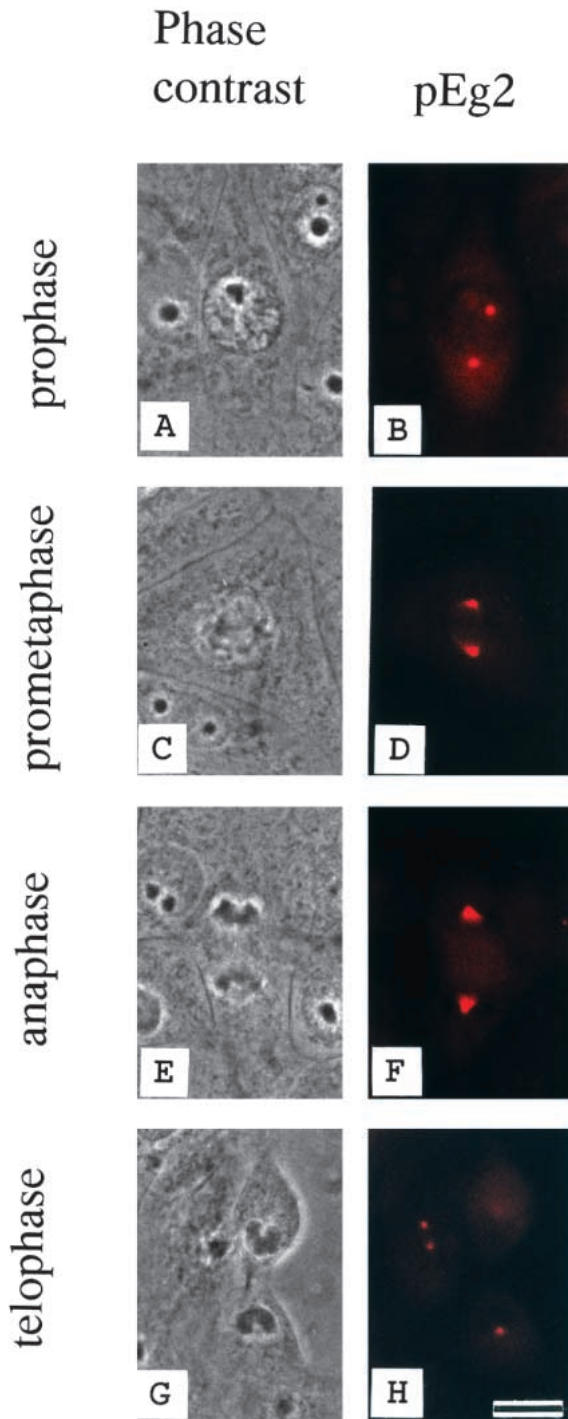
Fig. 6. Immunolocalisation of pEg2 at different mitotic stages. The monoclonal anti-pEg2 antibody (1C1) was used to immunolocalise pEg2 in *Xenopus* XL2 cells at different stages of mitosis. Cells were observed using phase contrast microscopy (A,C,E,G) to determine the stage of mitosis. Cell in A and B is in prophase; in C and D in prometaphase; in E and F in anaphase and in G and H in telophase Cells were labelled for indirect immunofluorescence microscopy using anti-pEg2 1C1 monoclonal antibody (B,D,F,H). Bar, 10 μm.

pEg2 present in the cytosol was found in the microtubule pellet (Fig. 8B, lower panel, lane 4). This result shows that pEg2 binds to microtubules, but does not demonstrate a physical interaction between pEg2 and tubulin. Association of pEg2 with the microtubules may occur directly or through an interaction with a microtubule-associated protein. To discriminate between those two possibilities we polymerised microtubules from pure bovine tubulin in the presence of recombinant pEg2-(His)6 protein. In this experiment, pEg2-(His)6 molecules were only found in the pellet (Fig. 8C, lane 1), demonstrating that pEg2-(His)6 associated directly with microtubules. pEg2-(His)6-microtubule cosedimentation can be inhibited by addition of salt, confirming an electrostatic interaction between pEg2 and microtubules (Fig. 8C, lane 3). In contrast, addition of ATP does not cause the release of pEg2 from the microtubules (data not shown).

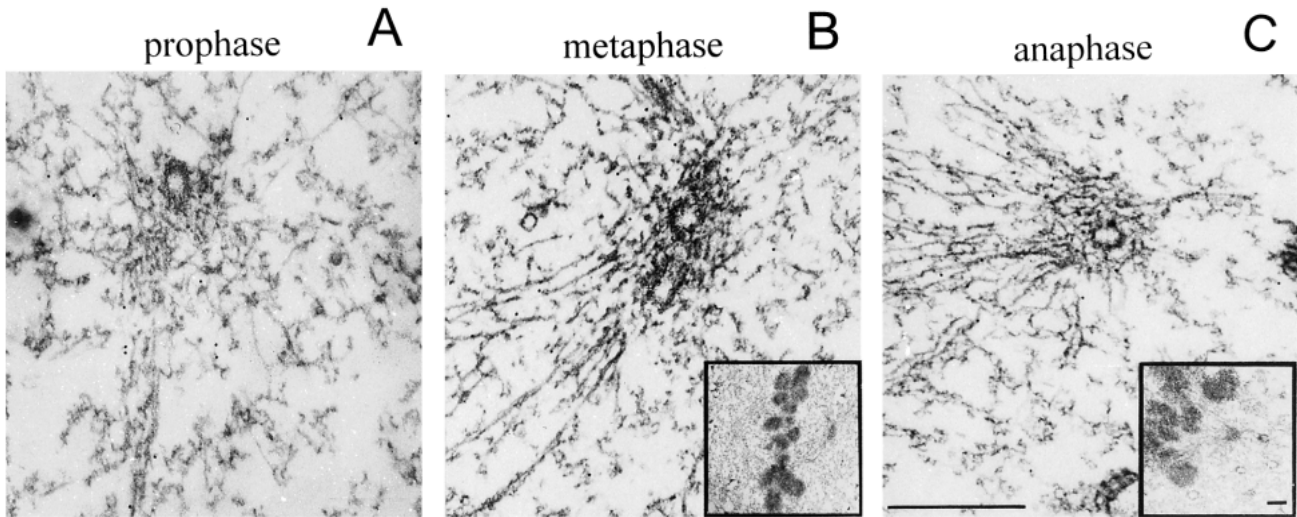## Binding of pEg2 to microtubules is not required for its centrosome association

In light of the results showing that pEg2 can bind to microtubules, we investigated whether pEg2 could stay associated with the centrosome in mitotic XL2 cells, once microtubules had been chemically depolymerized. *Xenopus* XL2 cells were treated for 6 hours in the presence of 10 μg/ml nocodazole and stained with an anti-β-tubulin monoclonal antibody to assess microtubule depolymerisation. Two foci found within the cytoplasm of all mitotic cells observed, corresponded to the two duplicated centrosomes (Fig. 9B). Mitotic XL2 cells treated with nocodazole were also double-stained with anti-pEg2 1C1 monoclonal antibody and anti-γ-tubulin polyclonal antibodies. We found that pEg2 staining (Fig. 9E) coincided with the foci of staining observed with γ-tubulin antibody (Fig. 9D). Our data show that pEg2 remains associated with the centrosome in the absence of microtubules, suggesting an association of pEg2 with centrosomal material.

pEg2 binds only to bipolar mitotic spindle microtubules

To examine whether or not pEg2 has an effect on microtubule nucleation, we treated mitotic XL2 cells with taxol to stabilise the microtubule array. Staining of the cell with an anti-β-tubulin antibody revealed the formation of several miniasters of microtubules in the cytoplasm, and bipolar spindles were never detected (Fig. 10B). As revealed using an anti-γ-tubulin antibody, only two of these asters contained a centrosome (Fig. 10D) (Novakova et al., 1996; Komarova et al., 1995). This observation suggests that a fraction of nucleating material has probably been redistributed throughout the cytoplasm in the cell to reconstitute cryptic MTOC (microtubule organising center) activity.
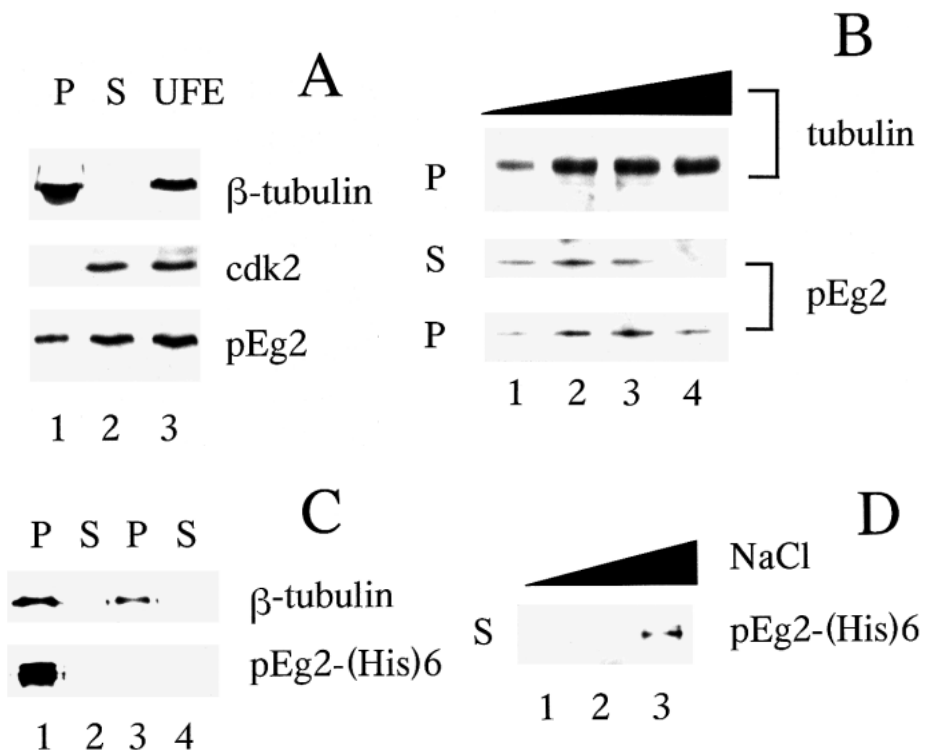
Mitotic cells containing multiple miniasters were also stained with the anti-pEg2 monoclonal antibody, and interestingly pEg2 was only found in the two asters containing a centrosome (Fig. 10E), and was undetectable in the center of miniasters lacking centrosomes. Furthermore, pEg2 was not detected on the microtubules forming any of these asters. pEg2 cup-shaped staining, characteristic of bipolar spindles in untreated cells, was never observed in taxol-treated cells, even on those asters containing a centrosome.

These results suggested that the relocalisation of pEg2 to the poles of the spindle during metaphase does require microtubules but also requires an intact bipolar spindle. In

of increasing amount of exogenous bovine brain tubulin (Fig. 8B). When microtubule polymerisation was performed in 100 μl of egg cytosol containing 120 μg of purified tubulin, all

Fig. 7. Ultrastructural localisation of pEg2 in mitotic cells. *Xenopus* XL2 cells were grown on coverslips, fixed in cold methanol (−20°C), incubated with the monoclonal antibody 1C1 and processed for electron microscopy as described in Materials and Methods. pEg2 has been detected using the 1C1 monoclonal antibody and revealed with a secondary antibody coupled to 15 nm gold particles. (A) A centrosome in prophase, (B) enlargement of the right pole of the spindle in metaphase (the metaphase plate is shown in the panel in the bottom right corner) and (C) one pole of a spindle in anaphase (the position of chromosomes is shown in the lower panel). Bars: 1 μm (A, B and C); 0.5 μm (insets in B and C).

Fig. 8. pEg2 binds to microtubules. Western blot analysis of microtubule co-pelleting assays. In all experiments, the pellet was resuspended in a volume of Laemmli sample buffer identical to the volume of the supernatant fraction to allow comparisons of protein concentration. (A) Microtubules were polymerised in *Xenopus* egg metaphase cytosol in the presence of GTP and taxol. Incubations were centrifuged and separated into pellet (lane 1) and supernatant (lane 2). Total proteins from one egg (UFE) were loaded in lane 3 for comparison. Proteins were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and probed with various antibodies. Upper panel, anti-β-tubulin monoclonal antibody; middle panel, anti-cdk2 antibody; lower panel, anti-pEg2 1C1 monoclonal antibody. (B) Microtubules were polymerised in the conditions used in A in the absence (lane 1) or presence of 20 μg (lane 2), 40 μg (lane 3) and 120 μg (lane 4) of exogenous bovine tubulin. Mixtures were centrifuged and separated into pellet (P) and supernatant (S), separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with anti-β-tubulin monoclonal antibody (upper panel) and anti-pEg2 1C1 monoclonal antibody (middle and lower panels). (C) Taxol-stabilised microtubules were polymerised in vitro from 37.5 μg of purified bovine tubulin in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 2 μg of recombinant pEg2-(His)6. Reactions were centrifuged and separated into pellet (P) and supernatant (S), separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with anti-β-tubulin monoclonal antibody (upper panel) and anti-pEg2 1C1 monoclonal antibody (lower panel). (D) Binding of pEg2-(His)6 to microtubules is inhibited by salt. Taxol-stabilised microtubules were polymerised in vitro from 37.5 μg of purified bovine tubulin in the presence of 2 μg of recombinant pEg2-(His)6 in absence (lane 1) or in presence of 500 mM (lane 2) and 1 M (lane 3) of NaCl. The supernatant (S, not shown) and the pellet (P) fractions were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and probed with the anti-pEg2 1C1 monoclonal antibody.
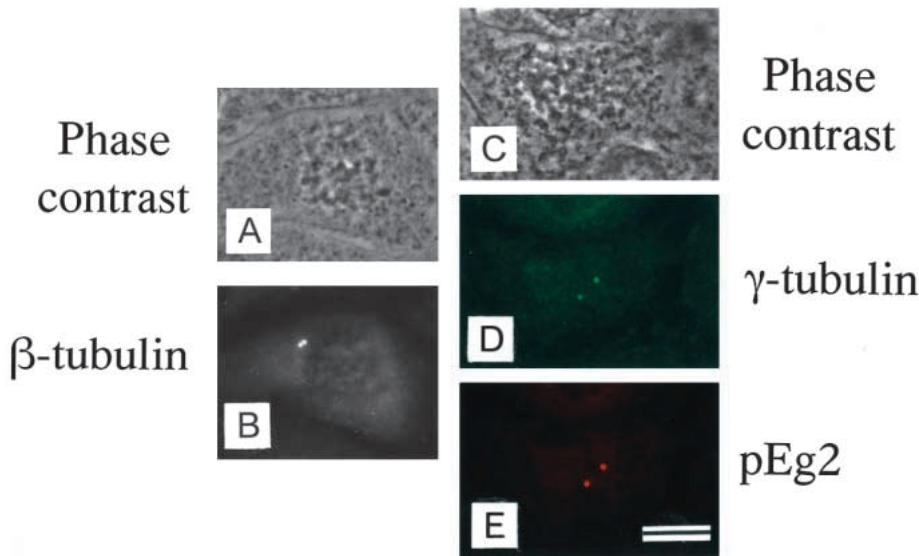
Fig. 9. Depolymerisation of mitotic microtubules does not affect pEg2 centrosome localisation in mitosis. After 48 hours in culture, XL2 cells were incubated for 6 hours with 10 µg/ml nocodazole, fixed and labeled for indirect immunofluorescence microscopy with various antibodies: anti-β-tubulin monoclonal antibody (B); anti-γ-tubulin rabbit polyclonal antibody (D); and anti-pEg2 1C1 monoclonal antibody (E). Stages of the cell cycle were determined using phase contrast microscopy (A and C). The cell shown in A and B is different from the one presented in C, D and E. Both cells are in mitosis with condensed chromosomes. pEg2 is still localised at the centrosome after the chemical-induced depolymerisation of mitotic microtubules. Bar, 10 µm.

addition, the absence of pEg2 on microtubule asters suggests that the kinase activity is not required for nucleation of microtubules. However, a role in the dynamics of microtubule nucleation might certainly be considered.

Eg2 activity is required for bipolar spindle assembly

The events that occur during cell cycle progression, such as spindle assembly, can be easily monitored in vitro using CSF-arrested *Xenopus* egg extracts. Incubation of demembranated sperm nuclei in CSF extracts in the presence of calcium results in the reformation of nuclear envelopes around the decondensed chromatin. Subsequently, the nuclei enter S phase and replicate their DNA (Blow and Laskey, 1986). If at that stage the interphasic extract (containing nuclei, which have completed DNA replication) is supplemented with half a volume of fresh CSF-arrested extract, it continues its progression through the cell cycle and then arrests in metaphase. A typical experiment is presented in Fig. 11B, panels 1 to 4. Nuclei that have replicated their DNA and interphasic microtubules are shown

in Fig. 11B, panels 1 and 2, respectively. When fresh CSF-arrested extract was added, the interphasic microtubule network depolymerised and mitotic bipolar spindles were formed (Fig. 11B, panels 3 and 4).

If pEg2 is involved in this process, inhibition of pEg2 kinase activity should specifically interfere with spindle assembly. We engineered a catalytically inactive form of the kinase by replacing lysine 169 located in the ATP binding site of pEg2-(His)6 with an arginine. The mutated recombinant protein named pEg2-K/R-(His)6, was over-expressed in *E. coli* and purified on a nickel affinity column (Fig. 11A, lane 1). The inactive protein migrated faster on SDS-polyacrylamide gel than the active protein and as a single band (Fig. 11A, lane 2), supporting the suggestion that the series of bands observed for active pEg2-(His)6 was due to autophosphorylation (Fig. 11A, lane 1 and Fig. 3A, lane 4). As expected, pEg2-K/R-(His)6 was unable to phosphorylate MBP or to autophosphorylate (Fig. 11A, lane 4) and thus could behave like a dominant negative mutant (Mandenhall et al., 1988). Purified inactive pEg2-K/R-
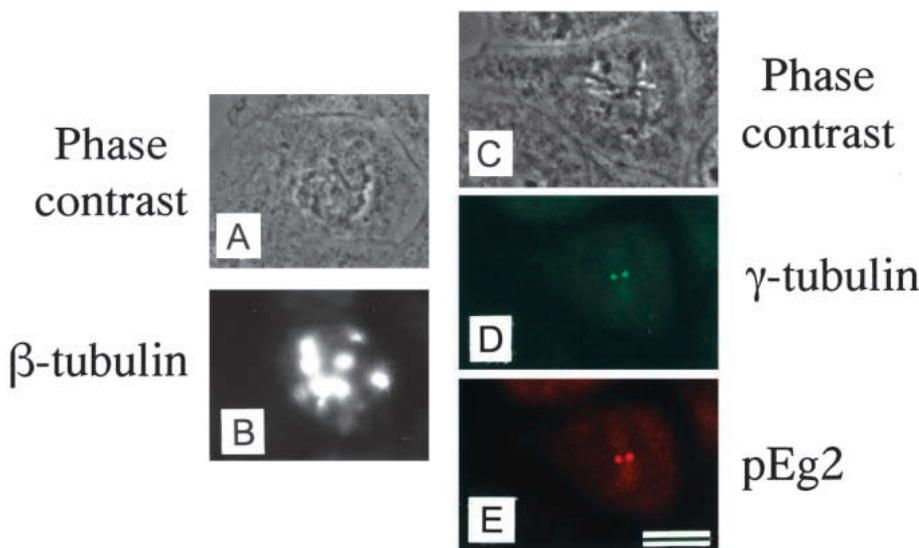
Fig. 10. pEg2 associates only with microtubule asters containing centrosomes. XL2 cells were treated with (10 µg/ml) taxol to induce the formation of multiple asters in the cell cytoplasm. Cell were fixed and labeled for indirect immunofluorescence microscopy with various antibodies: anti-β-tubulin monoclonal antibody (B); anti-γ-tubulin rabbit polyclonal antibody (D); and anti-pEg2 1C1 monoclonal antibody (E). Cells were also observed using phase contrast microscopy (A and C). The cell shown in A and B is different from the one presented in C, D and E. pEg2 is localised only in the center of the asters containing centrosomes. Bar, 10 µm.
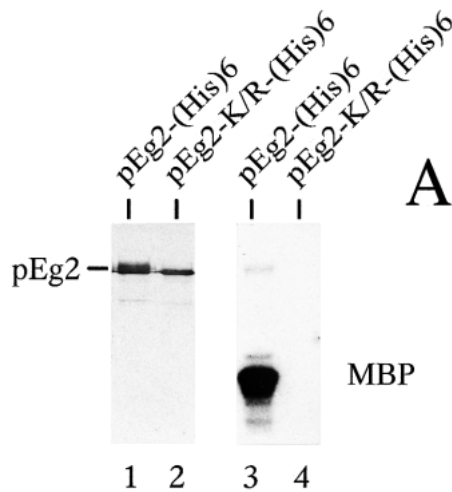
Fig. 11. Inhibition of bipolar mitotic spindle formation in *Xenopus* egg extract by addition of a dominant negative mutant protein pEg2-K/R-(His)6. (A) Protein kinase activity of the purified Eg2 recombinant proteins. Phosphorylation reactions were performed at 37°C in kinase buffer (see Materials and Methods) in the presence of 10 μM ATP containing 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmole) and 5 μg of MBP. At the end of the reaction samples were fractionated by electrophoresis on a 17.5% SDS-polyacrylamide gel. After transfer, the nitrocellulose membrane was used for immunodetection (left panel) and autoradiographed (right panel). Lanes 1 and 3, pEg2-(His)6; lanes 2 and 4, pEg2-K/R-(His)6. (B) In vitro spindle assembly. Reactions were performed in fresh CSF extract containing rhodamine-labelled tubulin and demembranated sperm heads. Nuclei were formed 45 minutes after addition of calcium. Extracts were then driven into metaphase and arrested by the addition of new CSF-arrested extract. Reactions were followed by fluorescence microscopy. Even numbered panels show DNA staining with Hoechst, odd numbered panels show microtubules visualised by rhodamine-labelled tubulin.. Panels 1 and 2, nuclei and microtubules obtained after incubation of demembranated sperm nuclei incubated in CSF-arrested egg extract for 15 minutes following by a further 45 minutes incubation in the presence of calcium. Panels 3 and 4, metaphase plate and mitotic spindle obtained 35 minutes after addition of CSF-arrested extract containing active pEg2-(His)6. Panels 5 to 10, DNA and microtubules observed after addition of CSF containing pEg2-K/R-(His)6. Panels 5 and 6, 35 minutes; panels 7 and 8, 45 minutes and panels 9 and 10, 55 minutes after the addition of CSF-arrested extract containing the inactive kinase.
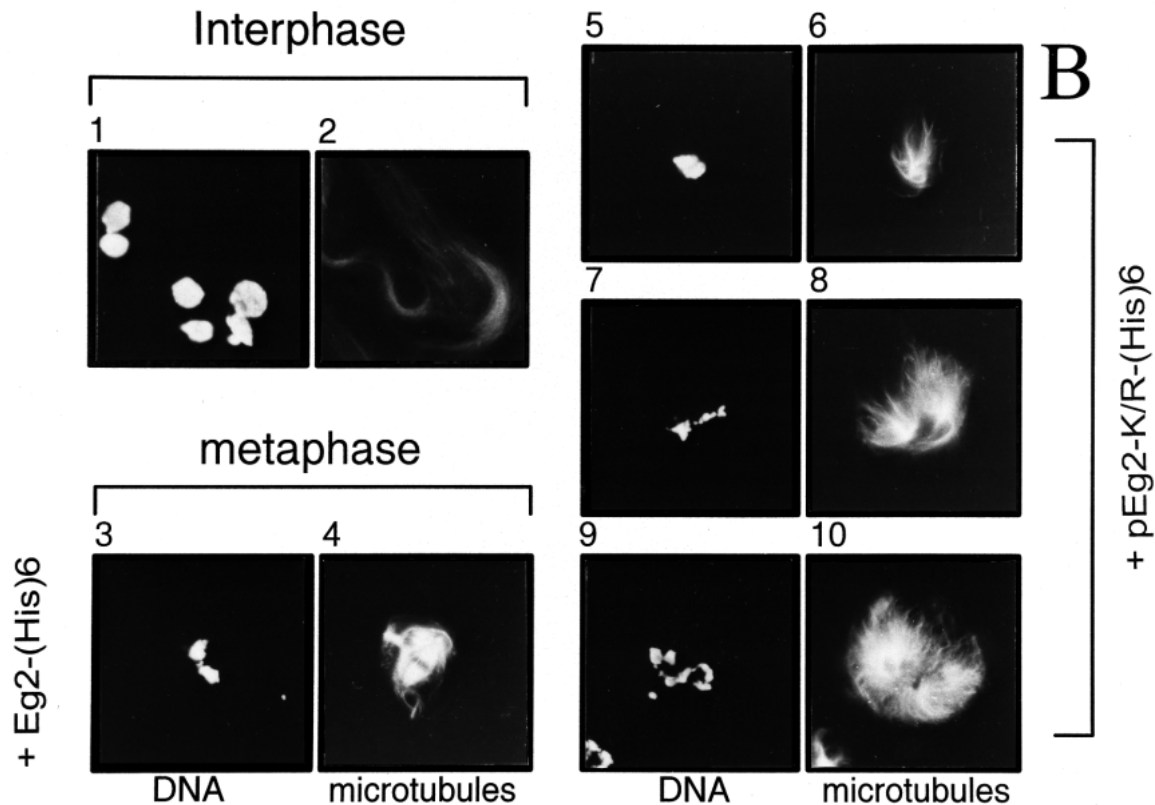
(His)6 or purified active pEg2-(His)6 were added to fresh CSF-arrested extracts which were then added to extract containing interphase nuclei. Addition of active pEg2-(His)6 had no detectable effect on the formation of mitotic bipolar spindles (Fig. 11B, panels 3 and 4). In contrast, extracts to which the inactive kinase was added formed only monopolar spindles (Fig. 11B, panel 6). The microtubules of these monopolar spindles kept growing throughout the experiment (Fig. 11B, panels 6, 8 and 10). These data suggest that pEg2 kinase is required for assembly of bipolar mitotic spindles, either in the establishment or maintenance of the bipolar spindle in *Xenopus* egg extracts. Presumably the dominant negative effect of the mutated kinase is due to titration of partner(s) and/or substrate(s) of endogenous pEg2.

DISCUSSION

A new and growing sub-family of protein kinases

pEg2 is a new member of a growing sub-family of protein kinases which includes Aik from human (Kimura et al., 1997), STK-1 (Niwa et al., 1996), IAK1 (Gopolan et al., 1997) and Ayk-1 (Yanai et al., 1997) from *M. musculus*, Aurora from *D. melanogaster* (Glover et al., 1995) and Ipl1 from *S. cerevisiae*

(Francisco et al., 1994). Most of these proteins are still only regarded as putative serine/threonine kinases. No associated protein kinase activity has yet been reported for STK-1, Ayk-1, Aurora and Ipl1 whereas Aik phosphorylates casein in vitro (Kimura et al., 1997) and IAK is able to phosphorylate MBP (Gopolan et al., 1997). In vitro, bacterially expressed pEg2-(His)6 also phosphorylates β-casein, but it phosphorylates MBP with much greater efficiency. It would be interesting to know if Aik is also able to phosphorylate MBP in addition to casein.

No biological role has yet been described for STK-1, Ayk-1, IAK1 or Aik, whereas Aurora and Ipl1 have been reported to be involved in centrosome migration (Glover et al., 1995) and in chromosome segregation, respectively (Chan and Botstein, 1993; Francisco et al., 1994). The localisation of pEg2 and its affinity for mitotic microtubules are consistent with a possible function for pEg2 either in centrosome migration, mitotic spindle assembly or stability, and/or chromosome segregation. To clarify the role of pEg2, we are currently investigating those different hypotheses using mitotic spindle assembly assays in *Xenopus* egg extracts (Murray and Kirschner, 1989).

The presence of pEg2 in the centrosome and at the poles of the mitotic spindle provide clues to its function

Immunofluorescence experiments in XL2 cells revealed that pEg2 partially colocalise with γ-tubulin in the centrosome during cell cycle progression. This localisation has been detected from S to M phase. Our attempts to localise pEg2 in cells in G_0 and G_1 phases have not been successful, presumably because the protein is present at low levels in these cells. We are now currently setting up conditions to synchronise *Xenopus* cultured cells in order to measure the amount of pEg2 precisely at each stage of the cell cycle. We have found a sequence in pEg2 that resembles a destruction box for the ubiquitination pathway, as has been reported for Aik (Kimura et al., 1997), which may explain the absence of pEg2 from G_1 cells.

In mitosis, the pattern of pEg2 staining (cup shaped) is more complex than the one observed during interphase (dot shaped). pEg2 is detected at the centrosome throughout mitosis. Depolymerisation of microtubule in mitosis does not alter the pEg2 centrosomal location suggesting that the kinase is associated with the pericentriolar material (PCM). The precise localisation observed following immunoelectron microscopy has first confirmed our immunofluorescence data and has also provided a detailed picture of pEg2 location in the centrosomal region. In prophase pEg2 localises around the PCM, in metaphase pEg2 seems to slide from its centrosome position to the microtubules at the poles of the bipolar spindle. In anaphase pEg2 seems to bind to the microtubules. Whether or not pEg2 is recruited onto microtubules is still unknown.

We have never detected pEg2 on astral microtubules by indirect immunofluorescence microscopy nor immunoelectron microscopy. This observation suggests that pEg2 may be able to make a clear distinction between the spatial orientations of the microtubules around the centrosome. Also pEg2 has not been detected on microtubules radiating from taxol induced asters in mitosis. This specific affinity for microtubules of the bipolar spindle may indicate that pEg2 is involved in the dynamics of spindle participation in chromosome segregation.

Localisation of two pEg2-related proteins has been already reported. Aik1 and IAK1 both have a centrosomal sub-cellular localisation, and they both slide on microtubules of the bipolar spindle. The main difference from pEg2 is that these proteins have been immunodetected only in mitosis (Kimura et al., 1997; Gopolan et al., 1997) whereas pEg2 was detected earlier in the cell cycle as well.

pEg2 binds to mitotic microtubules

Immunolocalisation of pEg2 in XL2 cells has provided an interesting piece of information regarding the possible function of the protein: pEg2 may bind to mitotic microtubules in vivo. We have shown that bacterially expressed pEg2-(His)6 fusion protein, as well as the endogenous protein are able to bind MTs in vitro. Direct binding of pEg2 to microtubules was a surprising finding because the kinase does not contain any known microtubule binding sequences, although a few repeats of basic residues (Noble et al., 1989) are found in the pEg2 sequence. To date, none of the pEg2-related kinases have been reported to bind to microtubules in vitro, although similar re-localisation to the poles of the spindle has been described in mitosis for Aik and IAK-1 (Kimura et al., 1997; Gopolan et al., 1997). Other protein kinases such as c-*mos* (Zhou et al., 1991) and ζPKC (Garcia-Rocha et al., 1997) have been previously reported to bind to microtubules but why these kinases are associated with microtubules is still unknown.

The sequences responsible for the binding of pEg2 to microtubules are under investigation, their identification will allow us to clarify the relationship between the microtubule binding and the kinase functions of pEg2. However, we need to keep in mind that pEg2 may require a partner to bind specifically to microtubules at the spindle pole.

Phosphorylation and the mitotic spindle

Phosphorylation appears to be a mechanism for the physiological control of the microtubule nucleating capability of the centrosome at mitosis. Among the large number of centrosome components, several are phosphoproteins (Vandré et al., 1984), protein kinases and protein phosphatases. Protein kinase activities play a central role in the control of the transition between interphase and mitosis (Karsenti et al., 1984; Verde et al., 1992). For example, phosphorylation by p34^{cdc2} increases the dynamics of microtubule nucleation (Ohta et al., 1993). It seems unlikely that the function of pEg2 at the centrosome is related to microtubule nucleating activity because centrosome-free asters (without detectable pEg2) are still able to nucleate microtubules efficiently.

pEg2 mitotic relocalisation on the microtubules at the poles of the spindle occurs only during metaphase and anaphase, suggesting that pEg2 may function in the bipolar spindle. Because of its sub-cellular localisation, we investigated the role of pEg2 in mitotic spindle assembly. We chose an in vitro assay using *Xenopus* egg extracts (Murray and Kirschner, 1989) and a dominant negative approach (Boleti et al., 1996). Addition of this purified mutant protein to *Xenopus* egg extracts that normally assemble bipolar mitotic spindles prevented bipolar spindle formation: instead only monopolar spindles were observed. Presumably a protein phosphorylated by pEg2 is required to ensure the bipolarity of the spindle either during the establishment or the maintenance of the bipolar spindle.

It has been reported that phosphorylation modulates the

affinity of microtubule binding proteins for the microtubule lattice. For instance, phosphorylation by p34[cdc2] increases microtubule-binding efficiency of the kinesin-related protein p135[Eg5] (Blangy et al., 1995), and decreases the affinity of the microtubule-associated protein MAP-2 (Faruki et al., 1992). Finding in vivo substrate(s) of the pEg2 kinase will provide clues to its function. Localization of pEg2 at the centrosome and its ability to bind to microtubules has already led us to search for possible substrates such as tubulin subunits, centrosome-associated proteins, microtubule-binding proteins and microtubule motors. Preliminary data have shown that neither α, β nor γ-tubulin are in vitro substrates for pEg2. Immunoprecipitation experiments have also been undertaken to identify proteins that associate with pEg2. Several high molecular mass proteins that are highly and specifically phosphorylated by pEg2 have been detected and these will be identified.

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