The non-catalytic domain of the *Xenopus laevis* auroraA kinase localises the protein to the centrosome

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SUMMARY

Aurora kinases are involved in mitotic events that control chromosome segregation. All members of this kinase subfamily possess two distinct domains, a highly conserved catalytic domain and an N-terminal non-catalytic extension that varies in size and sequence. To investigate the role of this variable non-catalytic region we overexpressed and purified *Xenopus laevis* auroraA (pEg2) histidine-tagged N-terminal peptide from bacterial cells. The peptide has no effect on the in vitro auroraA kinase activity, but it inhibits both bipolar spindle assembly and stability in *Xenopus* egg extracts. Unlike the full-length protein, the N-terminal domain shows only low affinity for paclitaxel-stabilised microtubules in vitro, but localises to the centrosomes in a microtubule-dependent manner. When expressed in *Xenopus* XL2 cells, it is able to target the green fluorescent protein to centrosomes. Surprisingly, this is also true of the pEg2 catalytic domain, although to a lesser extent. The centrosome localisation of the N-terminal peptide was disrupted by nocodazole whereas localisation of the catalytic domain was not, suggesting that in order to efficiently localise to the centrosome, pEg2 kinase required the non-catalytic N-terminal domain and the presence of microtubules.

Key words: AuroraA, *Xenopus*, Centrosome, Spindle, Localisation

INTRODUCTION

Aurora serine/threonine kinases control multiple mitotic events necessary for proper chromosome segregation (Bishoff and Plowman, 1999; Giet and Prigent, 1999 for review). While only one kinase is present in yeast, three different aurora kinases have been found so far in mammalian cells (Francisco et al., 1994; Shindo et al., 1998; Prigent et al., 1998; Bernard et al., 1998). The three kinases fulfil different functions during mitosis. AuroraA is involved in centrosome separation, which is a prerequisite for the formation of the bipolar mitotic spindle (Glover et al., 1995; Roghi et al., 1998). The activity of this kinase is also necessary for maintaining the stability of the spindle (Giet and Prigent, 2000), presumably because it phosphorylates the kinesin-related protein XlEg5 (Giet et al., 1999). In *Xenopus* oocyte, auroraA (pEg2) has been reported to be the first kinase activated during progesterone-induced maturation (within 30 minutes) and to phosphorylate CPEB (cytoplasmique polyadenylation element binding protein) necessary for the polyadenylation and the translation of *cmos* mRNA (Andresson and Ruderman, 1998; Mendez et al., 2000). The situation needs to be clarified, however, because in our hands the kinase is not activated until the time of GVBD (Germinal Vesicle Break Down), which occurs several hours after progesterone activation (Franck-Vaillant et al., 2000). The human kinase auroraA is overexpressed in various human cancers and overexpression in cultured cells is sufficient to impart a transformed phenotype (Sen et al., 1997; Bischoff et al., 1998; Zhou et al., 1998; Tatsuka et al., 1998; Tanaka et al., 1999). Although the other two human kinases have also been found to be overexpressed in various cancer cells, the relationship between the cancer state of the cells and the overexpression of the kinase is unrelated (Tatsuka et al., 1998; Kimura et al., 1999).

AuroraB localises to the midbody and its activity is required for cytokinesis (Tatsuka et al., 1998; Terada et al., 1998; Bischoff et al., 1998; Shindo et al., 1998). In *C. Elegans* auroraB (Air-2) phosphorylation of histone H3 serine 10 is necessary for its mitotic functions (Hsu et al., 2000). Although H3 is the only known substrate of auroraB, many interacting proteins have been identified. AuroraB is a chromosome passenger, and is targeted to the central spindle by interacting with INCENP (Adams et al., 2000; Kaitna et al., 2000); its localisation depends also on the presence of a survivin-like protein (Speliotes et al., 2000) and it seems to interact with a mitotic kinesin-like protein involved in cytokinesis (Severson et al., 2000).

AuroraC also localises to the centrosome but only during anaphase, and its function remains to be determined (Kimura et al., 1999). Several studies have reported an exclusive germline expression for auroraC (Bernard et al., 1998; Tseng et al., 1998; Hu et al., 2000).

The three human kinases share a very conserved C-terminal catalytic domain but each of them possesses an N-terminal

domain that is different in size and in sequence (Giet and Prigent, 1999). The non-catalytic domains of protein kinases fulfil at least two functions in vivo: to regulate the kinase activity and to localise the protein. Regulation of the catalytic activity via the non-catalytic domain is found in calcium/calmodulin kinases (CaMK) (Parissenti et al., 1998; Pearson et al., 1988; Goldberg et al., 1996), protein kinase C (PKC) (House and Kemp, 1987; Newton, 1995; Parissenti et al., 1998; Makowske and Rosen, 1989) and polo-like kinases (PLK) (Mundt et al., 1997; Lee and Erikson, 1997). Non-catalytic domains of protein kinases also serve as localisation domains to target the catalytic activity to restricted areas of the cell with precise timing during cell cycle progression. The localisation domain can be an associated protein, as for cAMP-dependent protein kinases (PKA) (Chen et al., 1997; Miki and Eddy, 1998) and the cyclin-dependent kinases (cdk), (Ookata et al., 1995; Cassimeris et al., 1999; Jackman et al., 1995), or the non-catalytic domain of the kinase, as in, for example, the polo-like kinases (Glover et al., 1998; Lee et al., 1998; Arnaud et al., 1998; Song et al., 2000).

There is circumstantial evidence that the localisation of the aurora kinase proteins may depend upon the non-catalytic domain of the kinase. The expression of human auroraA in the yeast *Ipl1*^{ts} mutant aggravated the Ipl1 phenotype at the permissive temperature, whereas the *Ipl1*^{ts} phenotype was partially rescued by a hybrid kinase comprising the Ipl1p non-catalytic domain fused to the human auroraA catalytic domain (Bischoff et al., 1998). These results seem to indicate that the human kinase N-terminal domain cannot be used in yeast.

In this report, we have investigated the function of the non-catalytic domain of the *Xenopus laevis* auroraA kinase pEg2, which is the orthologue of the oncogenic human kinase auroraA (aurora2). We present evidence that the N-terminal domain is essential for the localisation of the kinase in the cell.

MATERIALS AND METHODS

Constructions of vectors

The sequence of the pEg2 N-terminal domain (Nt) was amplified by PCR using two sets of primers containing restriction sites (underlined) (1) 5′-CCCTATCTCGAGGTCTTCCAGGCACC-3′ (*Nhe*I), (2) 5′-ATGGCTAGCGAGCGGGCTGTTAAGGAGAACC-3′ (*Xho*I), (3) 5′-GTCGACGGTACCATAATGGAGCGGG-3′ (*Kpn*I) and (4) 5′-GGTTGGATCCCGGGAGTCTTCCAGGCACCATTG-3′ (*Sma*I). The sequence of the pEg2 catalytic-domain (Cd) was also amplified by PCR using the following primers containing restriction sites (underlined) (5) 5′-TCGACGGTACCGATAATGGAAGACTTTGA-AATAGGG-3′ (*Kpn*I) and (6) 5′-CGGTGGATCCCGGGCTTGGG-CGCCTGGAAGGGG-3′ (*Sma*I). The PCR products (*Nhe*I/*Xho*I) and (Nt-*Kpn*I/*Sma*I and Cd-*Kpn*I/*Sma*I) were directly introduced into pGEM-T Easy vector (Promega). The *Nhe*I/*Xho*I fragment was then cloned in the pET21a(+) bacterial expression vector (Novagen Inc.) and the *Kpn*I/*Sma*I fragments were cloned in pEGFPN1 mammalian expression vector (Clontech).

Antibodies

Two mouse monoclonal antibodies raised against pEg2-(His)6 were used. The 1C1 antibody recognises the endogenous pEg2 and the recombinant histidine-tagged protein, while the 6E3 antibody only detects the recombinant histidine-tagged proteins **(**Giet and Prigent, 1998). Both 1C1 and 6E3 antibodies were affinity purified on protein G-Sepharose beads (Amersham Pharmacia Biotech) using standard methods (Harlow and Lane, 1988). Antibodies fixed to the beads were washed with PBS (136 mM NaCl, 26 mM KCl, 2 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) and eluted with 100 mM glycine, pH 2.9, and the fractions were collected in tubes containing 0.1 volume of 1 M Tris/HCl, pH 10. The antibodies were then diluted in 10 mM Hepes, 100 mM KCl and 2 mM $MgCl_2$, and concentrated using a centricon 30 (Amicon) to 3-4 mg/ml. Antibodies were stored at –80°C. The rabbit anti-XlEg5 polyclonal antibody was a gift from Dr Anne Blangy. The rabbit anti-γ-tubulin polyclonal antibody was a gift from Dr Michel Bornens (Institut Curie, Paris, France). The mouse anti-β-tubulin monoclonal antibody (clone Tub2.1) was purchased from Sigma Chemicals.

Cell culture and transfection

For indirect immunofluorescence studies, *Xenopus* XL2 cells (embryonic cell line) (Anizet et al., 1981) were cultured on glass coverslips as previously described (Uzbekov et al., 1998). Cells were washed with PBS, fixed in cold methanol (6 minutes at –20°C) and stored at –20°C until used. For transfection, XL2 cells were subcultured on 22-mm diameter glass coverslips in 60-mm plastic Petri dishes, and transfection was carried out using Transfast transfection kit from Promega following the manufacturer's instructions. The cells were cultured for 36 hours post-transfection prior to fixation.

Purification of recombinant proteins

All recombinant proteins were expressed in *E. coli* strain BL21(DE3)pLysS. Histidine-tagged pEg2 proteins were purified on Ni-NTA-agarose beads (Qiagen S.A.) as described previously (Roghi et al., 1998) and the pMAL peptide was purified on amylose resin following the manufacturer's instructions (New England Biolabs). For use in spindle assembly and stability assays, proteins were diluted in 10 mM Hepes, 100 mM KCl and 2 mM $MgCl_2$, and concentrated using centricon 10 (Amicon) to 4 mg/ml. The proteins were then stored at –80°C.

Protein kinase assay

The assays were performed in 10 μl of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, and 10 μM ATP containing 0.5 μCi of [γ-^{32}P]dATP (3000 Ci/mmole; Amersham Pharmacia Biotech) containing 200 ng of pEg2-(His)6 and 4 μg of myelin basic protein (MBP) (Sigma Chemicals) in the presence of increasing amounts of either pEg2-K/R-(His)6 or Nt-pEg2-(His)6 proteins. The reactions were incubated at 37°C for 15 minutes, terminated by addition of 10 μl 2× Laemmli sample buffer (Laemmli, 1970) and heated at 90°C for 10 minutes. The proteins were then separated by SDS-polyacrylamide gel electrophoresis. The MBP band was cut out and the associated radioactivity determined by phosphoimager counting (Molecular Dynamics).

Affinity chromatography

The recombinant proteins were overexpressed in a 1 l bacterial culture and purified by affinity chromatography. The bacterial lysate was loaded onto a 200 μl Ni-NTA agarose column following the manufacturer's instructions (Qiagen S.A.). The column was extensively washed with PBS and loaded with 500 μl of *Xenopus* CSF extract prepared as previously described (Roghi et al., 1998). The column was again extensively washed with PBS, eluted using 250 mM imidazole and 200 μl fractions collected. 200 μl 2× Laemmli buffer was added to each fraction and heated for 10 minutes at 90°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane and identified by western blot analysis.

Western blot analysis

SDS-polyacrylamide gel electrophoresis and electrotransfer of proteins onto nitrocellulose were performed as previously described

(Roghi et al., 1998). 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 with the antibodies in TBST containing 2.5% skimmed milk for 1 hour at 4°C. Both 1C1 and 6E3 antibodies were used at 1:100 dilution and anti-XlEg5 at a dilution of 1:1000. Immunocomplexes were identified using either peroxidase or phosphatase-conjugated secondary antibodies (Sigma Chemicals) and chemiluminescence (Amersham Pharmacia Biotech), according to the manufacturer's instructions, or using NBT/BCIP (Sigma Chemicals) as the phosphatase substrate.

Microtubule co-pelleting assay

100 ng of purified recombinant protein was incubated for 30 minutes at 37°C in 50 μl of BRB80 (80 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8) containing 4 mM $MgCl_2$, 4 mM ATP, 4 mM GTP, 0.4 μg/μl bovine brain tubulin, 100 mM NaCl and 20 μM paclitaxel (in the control experiment the bovine brain tubulin was replaced by bovine serum albumin). After centrifugation at 37°C on a BRB80 glycerol cushion (BRB80, 50% glycerol, 10 μM paclitaxel, 2 mM GTP) at 100,000 ***g*** for 20 minutes, the pellets were resuspended in 60 μl 1× Laemmli sample buffer and 10 μl 6× Laemmli sample buffer were added to the 50 μl supernatant.

Co-pelleting assays were performed in 200 μl of high speed supernatant of *Xenopus* egg CSF extract containing ATPmix regenerator (10 mM creatine phosphate, 80 μg/ml creatine kinase, 2 mM ATP, 1 mM $MgCl_2$), 250 ng of Nt-pEg2-(His)6 and 20 μM of paclitaxel or 10 μg/ml nocodazole. After 30 minutes at 23°C, the reaction mixtures were centrifuged at 23°C through a BRB80 glycerol cushion at 100,000 ***g*** for 20 minutes and the pellets and supernatant fractions processed as described previously. The protein fractions were heated for 10 minutes at 90°C, separated by SDS-polyacrylamide gels electrophoresis, transferred onto nitrocellulose membranes and analysed by western blotting.

Spindle assembly and stability assay

Spindles were assembled as described previously (Roghi et al., 1998) using *Xenopus* egg CSF extracts. Briefly, sperm nuclei (200/μl) were incubated in a CSF extract (20 μl) for 15 minutes at 23°C and activated by the addition of $CaCl_2$ (0.4 mM). Bovine brain rhodamine-labelled tubulin from Tebu (0.4 μg/ml final concentration) was added to the extract to visualise the microtubules. After 60 minutes, the extract was driven into mitosis and arrested in metaphase by the addition of 20 μl of CSF extract containing 16 μg of purified Nt-pEg2-(His)6 (400 ng/μl final concentration) or 16 μg of pMAL peptide. Once metaphase plates had formed (60-70 minutes after addition of the CSF extract), samples were fixed and mounted in a solution containing 15 mM Pipes, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 7.4% formaldehyde, 5 mM $MgCl_2$, 50% glycerol and 1 μg/μl of bis-benzymide and a minimum of 100 nuclei were scored. Each experiment was repeated 3 times.

To examine bipolar spindle stability, 4 μg of the purified recombinant protein was added to 10 μl of a metaphase spindle containing extract. After 60 minutes of incubation, nuclei were fixed, mounted as described previously, and scored under a DMRXA fluorescence microscope; the images were acquired with a black and white camera and treated with a Leica-Q-Fish program.

Indirect immunofluorescence

Green fluorescent protein (GFP)-transfected cells were fixed for 10 minutes in 75% methanol, 3.7% formaldehyde, 0.5× PBS, washed for 2 minutes with PBS containing 0.1% Triton X-100 and incubated for 1 hour in PBS containing 3% BSA at 20°C. Cells were incubated with the rabbit anti-γ-tubulin antibodies (dilution 1:1000) for 1 hour at 20°C in PBS containing 1% BSA. γ-tubulin was detected as described before using Texas Red-conjugated goat anti-rabbit antibody from Sigma Chemicals (diluted 1:1000 in PBS containing 1% BSA and 0.5 μg/ml Hoechst dye).

For immunolocalisation experiments in *Xenopus* extracts, the extracts were diluted 50 times in 80 mM Pipes, 2 mM $MgCl_2$, 2 mM EDTA, 15% glycerol, loaded onto 4 ml of the dilution buffer and centrifuged onto glass coverslips at 5000 ***g*** for 30 minutes at 23°C. After 6 minutes post-fixation in methanol at –20°C the coverslips were incubated in PBS containing 3% BSA for 1 hour at 4°C, followed by an incubation with 6E3 monoclonal (dilution 1:50) or 1C1 monoclonal (dilution 1:50) for 1 hour at 4°C in PBS containing 1% BSA (PBS/BSA). After incubation with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma Chemicals) in PBS/BSA containing 0.5 μg/ml Hoechst dye for 1 hour at 4°C, the reactions were mounted in PBS containing anti-fade and 50% glycerol and observed under a Leica DMRXA fluorescence microscope.

Centrosome binding assay in vitro

Sperm heads were incubated for 15 minutes at room temperature in mitotic extract (high speed supernatant) containing 400 ng/μl of Nt-pEg2-(His)6. Microtubule nucleation was monitored using rhodamine-labelled tubulin. Samples were diluted 50 times in BRB80 containing 15% glycerol, centrifuged onto glass coverslips, fixed for 6 minutes in cold methanol and processed for immunofluorescence as previously described. Endogenous pEg2 was detected with 1C1 monoclonal antibody and recombinant Nt-pEg2-(His)6 with 6E3 monoclonal antibody; Immunocomplexes were visualised using Texas Red-conjugated goat anti-mouse antibody. Centrosomes were detected with a rabbit anti γ-tubulin polyclonal antibody and with fluorescein isothyocyanate-conjugated goat anti-rabbit antibody. DNA was stained with Hoechst. The same experiment was repeated in the presence of 20 μM nocodazole.

RESULTS

The non-catalytic domain of the *Xenopus* pEg2 kinase

The N-terminal domains of the different vertebrate auroraA orthologues are similar (Fig. 1A). The N-terminal domain of the *Xenopus laevis* kinase shares 36% identity (50% similarity) with the human N-terminal domain and 32% identity (46% similarity) with the mouse N-terminal domain. The two mammalian kinase domains are more closely related, 64% of the amino acids are identical and they show 72% similarity. This N-terminal domain is also highly enriched in basic residues and has a pHi of 10.1. The fact that the domain has been conserved throughout evolution indicates that it may have a specific role and we investigated this function using the N-terminal domain of the *Xenopus laevis* pEg2 kinase. We first designed a recombinant histidine-tagged peptide [MASERAVKENHKP…EGKKKQWCLEHHHHHH], Nt-pEg2-(His)6, which we purified from *E. coli*. The peptide ends immediately before the start of the kinase catalytic domain [e.g. DFEIGRPLGKGK...]. The apparent molecular mass of the peptide on SDS-polyacrylamide gels was 22 kDa (Fig. 1B). To investigate the function of this domain we used the monoclonal antibodies 1C1 and 6E3 described previously (Giet and Prigent, 1998). They were both raised against the recombinant histidine-tagged pEg2 kinase. The 1C1 antibody detects both the endogenous and the recombinant kinase whereas the 6E3 antibody only detects the recombinant kinase (Giet and Prigent, 1998). Both antibodies behave similarly against Nt-pEg2(His)6 (Fig. 1C). The 1C1 antibody recognises both the endogenous pEg2 protein kinase (Fig. 1C, lane 2) and the Nt-pEg2(His)6 (Fig. 1C, lane 1). The 6E3

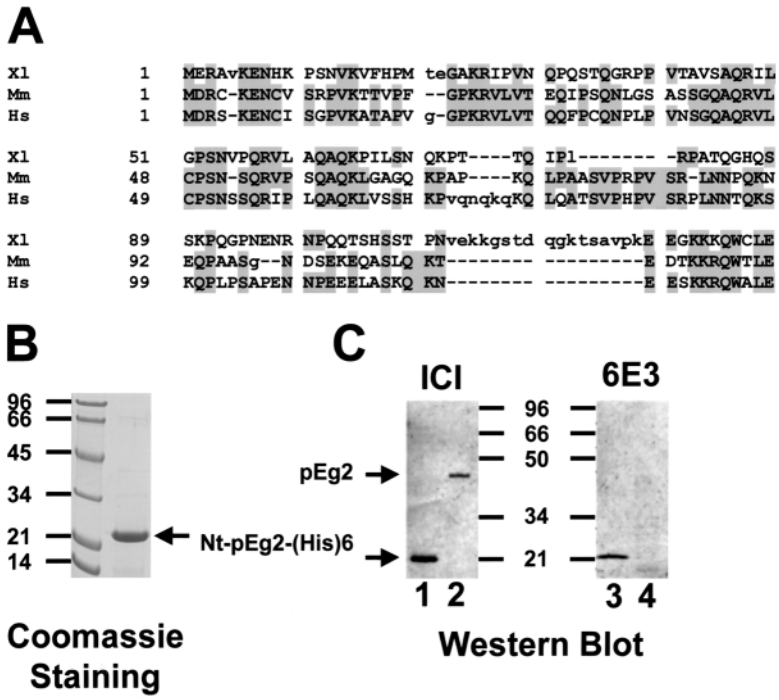

Fig. 1. Sequence comparison of vertebrate auroraA kinase non-catalytic domains, recombinant *Xenopus* auroraA N-terminal protein (Nt-pEg2-(His)6) and antibody specificity. (A) Sequence alignment of vertebrate auroraA N-terminal domains. Xl: *Xenopus laevis* pEg2, GenBank accession no. Z177207 (Roghi et al., 1998); Mm: *Mus musculus* AIR1, GenBank accession no. U69106) (Shindo et al., 1998); Hs: *Homo sapiens* aurora2, GenBank accession no. AF008551 (Shindo et al., 1998). Identical amino acids are shown in grey. (B) Nt-pEg2-(His)6 protein was overexpressed in *E. coli* and purified by affinity chromatography on a Ni-NTA agarose column. The protein was eluted with 250 mM imidazole and concentrated through a centricon 10. 2 μl (8 μg) were analysed on a 20% SDS-polyacrylamide gel stained with Coomassie Blue. (C) Specificity of 1C1 and 6E3 monoclonal antibodies. 1 μl of a *Xenopus* egg extract containing about 40 ng of endogenous pEg2 (lanes 2 and 4) or 40 ng of Nt-pEg2-(His)6 purified protein (lanes 1 and 3) were subjected to electrophoresis on a 20% SDS-polyacrylamide gel, transferred on nitrocellulose membrane and probed with 1C1 (lanes 1 and 2) or 6E3 (lanes 3 and 4) monoclonal antibodies (dilution 1/100). The positions of molecular mass (kDa) markers in B and C are shown.

antibody recognises only the recombinant Nt-pEg2-(His)6 protein (Fig. 1C, lane 3). This result indicates that the 1C1 antibody recognises an epitope present in both endogenous and recombinant protein kinases. The 6E3 antibody recognises an epitope, presumably in the amino-acid sequence of Eg2, that includes the sequence derived from the *Nhe*I site used for the cloning. This antibody does not recognise the catalytic domain of pEg2 cloned in the same *Nhe*I site, nor does it recognise other histidine-tagged proteins. This epitope is not present in the endogenous protein pEg2 (Fig. 1C, lane 4).

The N-terminal domain does not inhibit the kinase activity

We investigated whether the N-terminal domain of pEg2 has any regulatory effect on the kinase activity. The catalytic activity of the recombinant pEg2 kinase was assayed in vitro, in the presence of increasing amounts of the purified recombinant N-terminal domain, using the myelin basic protein (MBP) as a substrate. As a control for inhibitory activity we used the recombinant inactive form of pEg2. We have already shown that this recombinant inactive kinase pEg2-K/R-(His)6 behaves as a dominant negative form of the kinase in *Xenopus* extracts (Roghi et al., 1998; Giet and Prigent, 2000) and also as an inhibitor of pEg2 kinase activity in vitro (Giet et al., 1999). In the presence of increasing amounts of its N-terminal domain, the pEg2 kinase activity was not significantly reduced: 82% of the activity remained in the presence of 2 μg of the domain. In contrast, in the presence of 2 μg of the dominant negative form of pEg2, only 24% of the kinase activity was detected (Fig. 2A). The kinase activity of pEg2 is clearly not affected by the addition of the non-catalytic domain. As shown in Fig. 1B, the epitope of monoclonal antibody 1C1 maps to the N-terminal domain of the kinase. The kinase activity of recombinant pEg2 kinase is not affected by this antibody (Giet et al., 1999; Giet and Prigent, 2000). Furthermore, immunoprecipitated endogenous pEg2 remains active when attached to protein G-Sepharose beads (Giet et al., 1999). Together, these results indicate that the N-terminal domain of the kinase does not interfere with its catalytic activity. In agreement with this result we found that the N-terminal peptide does not associate with a known kinase substrate such as the kinesin-like protein XlEg5 (Fig. 2B, middle). This is not the case for either the full-length histidine-tagged pEg2 that binds to XlEg5 (Fig. 2B, top) or for the catalytic domain of pEg2, which associates to XlEg5 through the two-hybrid system (Giet et al., 1999).

The N-terminal domain of pEg2 has low affinity for paclitaxel-stabilised microtubules

We have previously shown that in vitro the intact kinase binds to paclitaxel-stabilised microtubules and that this binding is independent of its catalytic activity (Giet and Prigent, 1998), suggesting that the kinase may bind to microtubules through its non-catalytic domain. In order to test this suggestion, the ability of the N-terminal peptide to bind microtubules was determined.

Microtubules were polymerised in vitro from bovine brain tubulin in the presence of paclitaxel and recombinant proteins Nt-pEg2-(His)6 or pEg2-(His)6 (Fig. 3). After polymerisation, the reaction mixture was centrifuged through a glycerol cushion at 37°C. The pellet containing microtubules and microtubule associated proteins, and the supernatant containing proteins that have no affinity for the microtubules, were analysed for the presence of the recombinant proteins by western blotting. Control experiments were performed in which the bovine brain tubulin was replaced by bovine serum albumin (Fig. 3, lanes 1 and 2). In this case both the full-length kinase and the N-terminal domain remained in the soluble fraction (Fig. 3B,D, lane 1). When bovine brain tubulin was used the full-length kinase was detected only in the pellet fraction, indicating that it strongly associates with the

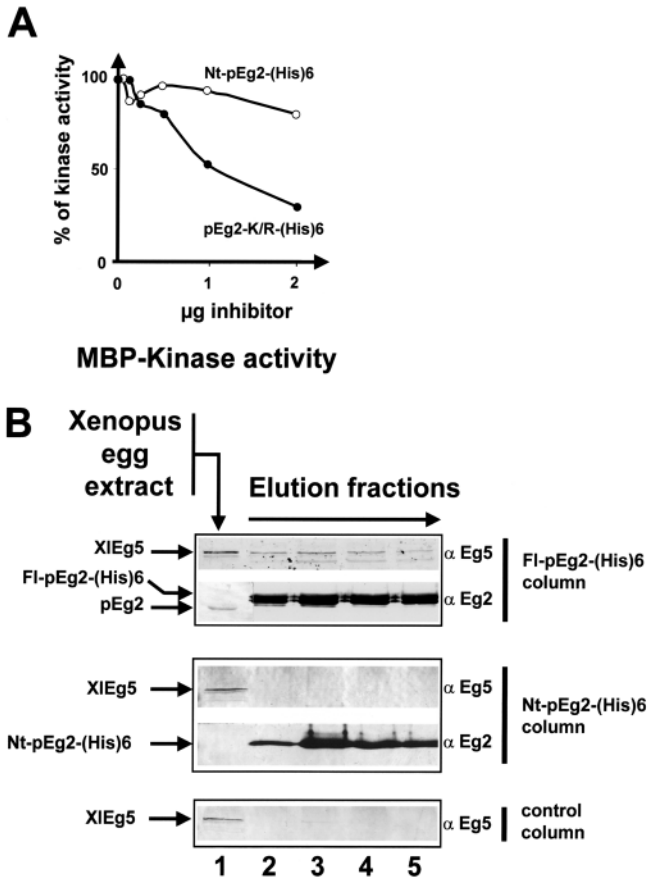

Fig. 2. The N-terminal domain of pEg2 does not affect the activity of the full-length kinase and does not bind to XlEg5. (A) The pEg2 kinase activity of the recombinant pEg2-(His)6 protein was assayed in vitro using myelin basic protein (MBP) as a substrate in the presence of increasing amounts of either the recombinant inactive pEg2-K/R-(His)6 kinase or the recombinant N-terminal domain Nt-pEg2-(His)6 protein. After incubation in the presence of [γ-^{32}P] ATP, the reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred onto a nitrocellulose membrane. The radioactive MBP was counted with a Phosphoimager. The kinase activity is expressed as a percentage of the activity without inhibitor. The kinase activity was estimated in the presence of the inactive kinase (filled circles) or the N-terminal domain of pEg2 (open circles). (B) Affinity chromatography. A Ni-NTA agarose column saturated with either Fl-pEg2-(His)6 (full length), Nt-pEg2-(His)6 (N-terminal) or no protein (control) was loaded with 200 µl of *Xenopus* CSF extract. Affinity-bound proteins in the different columns were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The membranes were cut and the upper part (>70 kDa) was incubated with anti-XlEg5 polyclonal antibodies (dilution 1/1000), while the lower part (<70 kDa) was incubated with the anti-pEg2 1C1 mAb (diluted 1/100). Lane 1, *Xenopus* egg extracts; lanes 2-5, elution fractions; Fl-pEg2-(His)6 column, nickel column preloaded with the full-length pEg2-(His)6 recombinant protein; Nt-pEg2-(His)6 column, nickel column preloaded with pEg2 N-terminal-(His)6 recombinant protein; Control column, control nickel column without any recombinant protein.

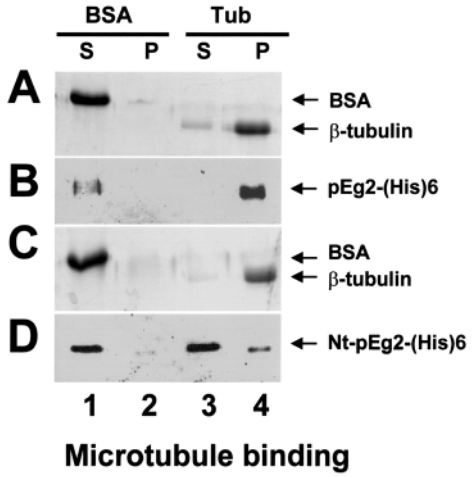

Fig. 3. The N-terminal domain of pEg2 shows much less affinity than the full-length protein for paclitaxel-stabilised microtubules in vitro. Microtubules were polymerised in vitro in the presence of purified bovine brain tubulin (lanes 3 and 4), stabilised with paclitaxel and centrifuged through a glycerol cushion to separate microtubules and microtubule associated protein (pellet, P) from proteins that do not associate to microtubules (supernatant, S). In a control reaction, purified tubulin was replaced by bovine serum albumin (lanes 1 and 2). In the presence of paclitaxel, the β-tubulin is recovered in the pellet (A and C, lane 4) whereas the bovine serum albumin remains in the supernatant (A and C, lane 1). Purified recombinant pEg2-(His)6 (A and B) or purified recombinant Nt-pEg2-(His)6 (C and D) were also incorporated to the reaction. The pellet (P) and the supernatant (S) were analysed for the presence of recombinant proteins using western blotting with the 1C1 monoclonal antibody (diluted 1:100).

microtubules (Fig. 3B, lane 4). In contrast, in the same conditions, only a small fraction of the N-terminal domain was found to be bound to the microtubules (Fig. 3D, lane 4) while a vast majority of the protein remains in the soluble fraction (Fig. 3D, lane 4).

The N-terminal domain of pEg2 binds to centrosomes assembled from sperm heads in *Xenopus* egg extracts

In light of the results showing that the Nt-pEg2-(His)6 protein had low affinity for microtubules we investigated whether the protein could associate with centrosomes. Centrosomes were assembled in vitro by incubating sperm nuclei in the high speed supernatant of *Xenopus* egg CSF extracts (Stearns and Kirschner, 1994) in the absence (Fig. 4A-H) or presence of the Nt-pEg2-(His)6 protein (Fig. 4I-P). Endogenous pEg2 was detected by 1C1 monoclonal antibody (Fig. 4C,G), Nt-pEg2-(His)6 with 6E3 monoclonal antibody (Fig. 4K,O), DNA with Hoechst (Fig. 4A,E,I,M) and γ-tubulin with a polyclonal antibody (Fig. 4B,F,J,N). After 15 minutes incubation of the sperm heads in the extract, both the endogenous pEg2 (Fig. 4C) and the γ-tubulin (Fig. 4B) were found to be associated with centrosome (Fig. 4D). When 400 ng/µl of the Nt-pEg2-(His)6 protein was added to the extract, the recombinant protein (Fig. 4K) and the γ-tubulin (Fig. 4J) localised to the centrosome. We then asked whether the Nt-pEg2-(His)6 would remain associated with centrosome in the presence of nocodazole. In other words, was the localisation microtubule-dependent? The endogenous pEg2 was found to remain associated with centrosomes in a microtubule-independent manner (Fig. 4G) just like γ-tubulin (Fig. 4F,N) (Felix et al., 1994; Stearns and

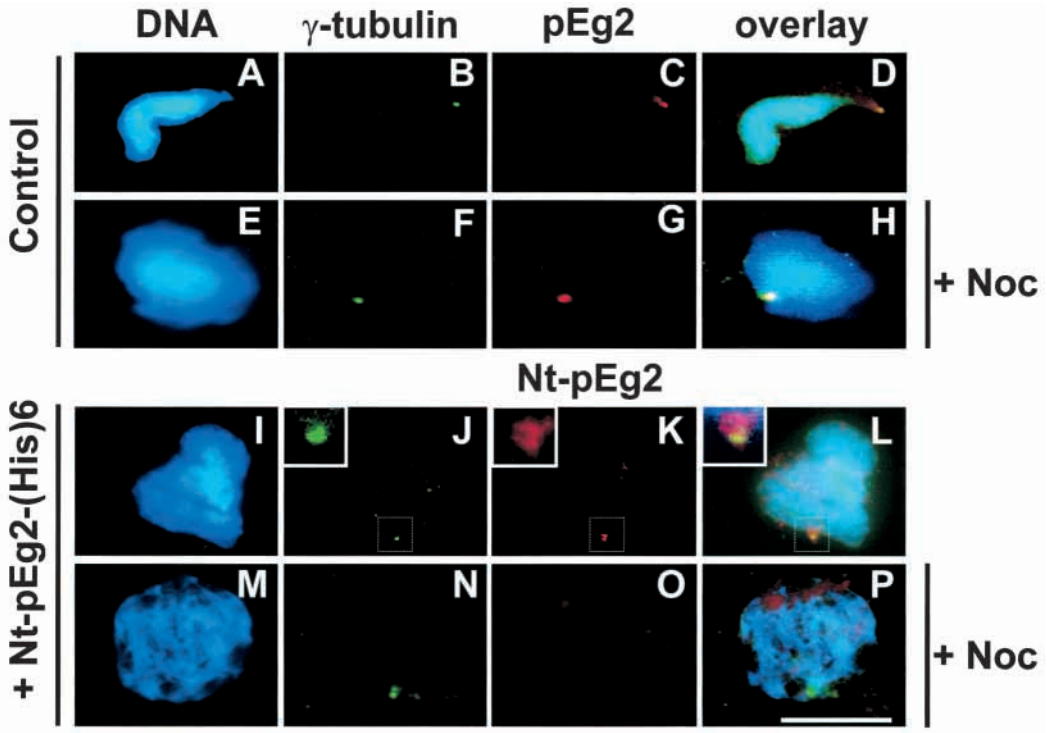

Fig. 4. The N-terminal domain of pEg2 binds to centrosomes assembled in *Xenopus* egg extract from sperm heads. Centrosomes were assembled in vitro by incubating demembranated sperm heads in *Xenopus* egg CSF extract (Stearns and Kirschner, 1994) in the absence (A-H) or presence (I-P) of 400 ng/μl of the recombinant Nt-pEg2-(His)6 protein. The reaction was performed in the absence (A-D and I-L) or presence of the microtubule depolymerising drug nocodazole (20 μM final concentration) (E-H and M-P). (A,E,I,M) Hoechst-stained DNA. (C,G) The endogenous pEg2 protein probed with mouse 1C1 monoclonal antibody (diluted 1:50). (K,O) The recombinant Nt-pEg2-(His)6 protein probed with mouse 6E3 monoclonal antibody (diluted 1:50). (B,F,JN) Centrosome stained with γ-tubulin antibody (diluted 1:1000). Fluorescein-conjugated anti-rabbit antibodies (diluted 1:500) and Texas Red-conjugated anti-mouse antibodies (diluted 1:500) were used as secondary antibodies. (D,H,L,P) Overlay. Scale bar, 10 μm. The localisation of the proteins was observed by fluorescence microscopy (DMRXA Leica); the images were acquired using a black and white camera and treated with the Leica-Q-Fish program.

Kirschner, 1994). In contrast, the association of the Nt-pEg2-(His)6 with the centrosomes was found to be microtubule-dependent (Fig. 4O). These results suggest that the N-terminal domain of pEg2 serves to localise the kinase to the centrosomes in a microtubule-dependent manner although this domain, by itself, has low affinity for microtubules.

In vivo, a pEg2 N-terminal domain GFP-fusion protein is targeted to the centrosomes in a microtubule-dependent manner

If the N-terminal domain of pEg2 is responsible for the localisation of the kinase, then fusion proteins containing the N-terminal peptide should localise to the centrosomes (Arnaud et al., 1998). We therefore fused the N-terminal domain of pEg2 to the N terminus of the GFP peptide (green fluorescent protein), inserted the construct into a expression vector under the control of a CMV (cytomegalovirus) promoter and transfected this into *Xenopus* XL2 cells. Transfected cells were easily discriminated from non-transfected cells because they expressed high levels of GFP. Centrosomes were detected using γ-tubulin indirect immunofluorescence. When the GFP protein alone was expressed, the whole cell was fluorescent, but GFP was concentrated in the nucleus (Fig. 5C, overexposure). The centrosomes, detected with anti-γ-tubulin staining, were not decorated with GFP (Fig. 5B,C).

In contrast, when the Nt-pEg2-GFP (Nt-GFP) protein was expressed in XL2 cells, the fusion protein localised to the centrosome (Fig. 5G). These results indicate that the N-terminal domain of pEg2 contains sufficient information to direct the localisation of GFP to the centrosome.

Because the catalytic domain of pEg2 was insoluble when expressed as a fusion protein in bacteria, we were unable to assay its affinity for the centrosome in *Xenopus* egg extract. We instead fused this catalytic domain to GFP (Cd-GFP) and expressed it in XL2 cells. Like the N-terminal domain, the catalytic domain was able to localise the GFP to the centrosomes (Fig. 5K). However a statistical analysis of the number of transfected cells containing GFP labelling of the centrosomes, revealed that there were about three times more cells containing Nt-GFP decorated centrosomes than Cd-GFP decorated centrosomes. This showed that, compared to the catalytic domain, there was a threefold increase in the efficiency of the N-terminal domain to localise the GFP to the centrosomes (Fig. 6, white bars).

Additionally, nocodazole treatment of transfected cells disrupted the centrosome localisation of the Nt-GFP whereas it had no effect on the localisation of the Cd-GFP (Fig. 6). This result indicates that the localisation of the Nt-GFP to the centrosomes depends upon the presence of microtubules, which is in agreement with the results obtained in *Xenopus* egg extracts. The localisation of the catalytic domain to the centrosomes may be due only to the affinity of the kinase for its substrates. Indeed

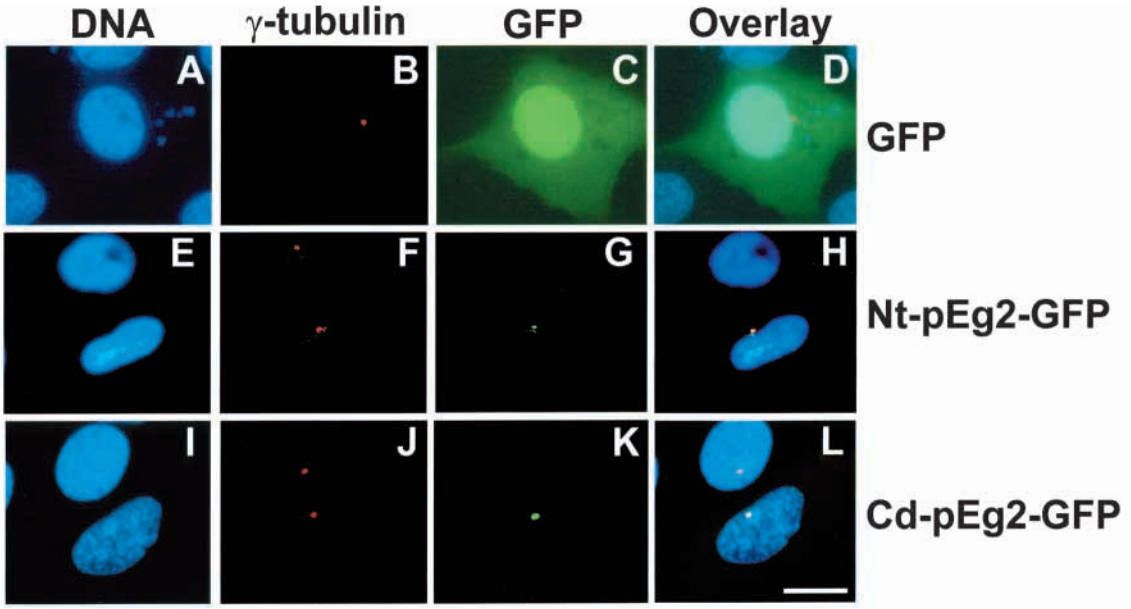

Fig. 5. The N-terminal domain of pEg2 fused to GFP localises to the centrosome in XL2 cells. GFP, Nt-pEg2-GFP and Cd-pEg2-GFP proteins were constitutively expressed in *Xenopus* XL-2 cells using transient transfection with the pEGFPN1 expression vector. Cells were fixed on coverslips and processed for immunofluorescence. (A-D) GFP transfected cells (control), (E-H) Nt-pEg2-GFP transfected cells, (I-L) Dc-pEg2-GFP transfected cells. (A,E,I) Hoechst-stained DNA. (B,F,J) γ-tubulin staining with a rabbit polyclonal antibody (diluted 1:1000), revealed with a Texas Red-conjugated secondary antibody (diluted 1:500). (C,G,K) The localisation of the GFP proteins. (D,H,L) Overlay. Scale bar, 10 μm. The localisation of the proteins was observed by fluorescence microscopy (DMRXA Leica); the images were acquired using a black and white camera and treated with the Leica-Q-Fish program

we have previously demonstrated using the two-hybrid system that pEg2 can interact with one of its substrates, XlEg5, through its catalytic domain (Giet et al., 1999).

The N-terminal domain of pEg2 inhibits bipolar spindle assembly and destabilises previously assembled bipolar spindles in *Xenopus* egg extracts

Clues about the function of the N-terminal domain of pEg2 came when we investigated the role of pEg2 in spindle assembly and spindle stability. We planned to use this domain as a control in the experiments and it turned out that in both assays the N-terminal domain of pEg2 shown a dominant negative effect. First we added the recombinant Nt-pEg2-(His)6 to the bipolar spindle assembly assay in *Xenopus* egg extract, as described previously (Roghi et al., 1998). The extract containing the N-terminal domain of pEg2 showed a decrease in the number of the bipolar spindles assembled. Instead of being bipolar (Fig. 7A, top), the majority of the abnormal spindles observed were monopolar (Fig. 7A, bottom). A quantitative measure of the assembly (Fig. 7B) clearly demonstrated an inhibitory effect of the N-terminal domain (34±18% of the spindles remained bipolar instead of 81±12% in the control).

We also found that the N-terminal domain of pEg2 destabilises previously assembled bipolar spindles. The majority of the abnormal structures produced upon addition of the N-terminal domain of pEg2 were also monopolar spindles. Hence the presence of the N-terminal peptide significantly reduces the stability of the spindles (55±5% of the spindles remained bipolar instead of 82±8% in the control) (Fig. 7C).

The N-terminal domain of pEg2 was not as efficient in inhibiting either the bipolar spindle assembly or spindle stability as the inactive form of the recombinant kinase, which allowed only 11% of the spindle to assemble bipolar structures and 14% of spindles to remain bipolar (Giet and Prigent, 2000). The effect of the N-terminal domain is comparable to the effect of the addition of the non-inhibitory anti-pEg2 1C1 mAb, which maps to an epitope in the N-terminal domain of the protein (Giet and Prigent, 2000). Because the N-terminal domain does not affect the kinase activity of pEg2 in vitro we assume that the dominant negative effect of the domain observed in the extracts is due to a localisation competition with the endogenous kinase.

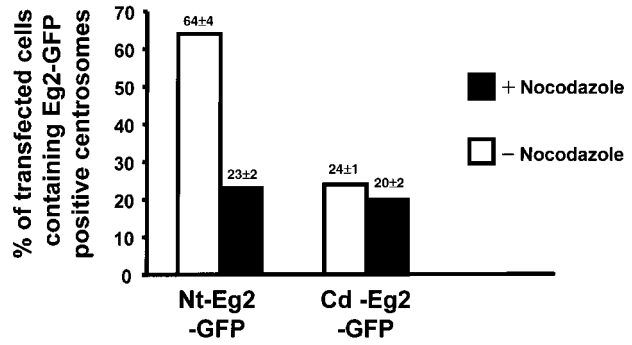

Fig. 6. The localisation of the pEg2 N terminus is microtubule-dependent. *Xenopus* XL2 cells expressing Nt-pEg2-GFP or Cd-pEg2-GFP protein were treated for 6 hours with the microtubule depolymerising drug nocodazole (10 μg/ml). The number of cells showing a centrosome localisation of the GFP fusion protein was then estimated. White bars, cells observed without nocodazole; black bars, cells after nocodazole treatment. Values are means ± s.e.m.

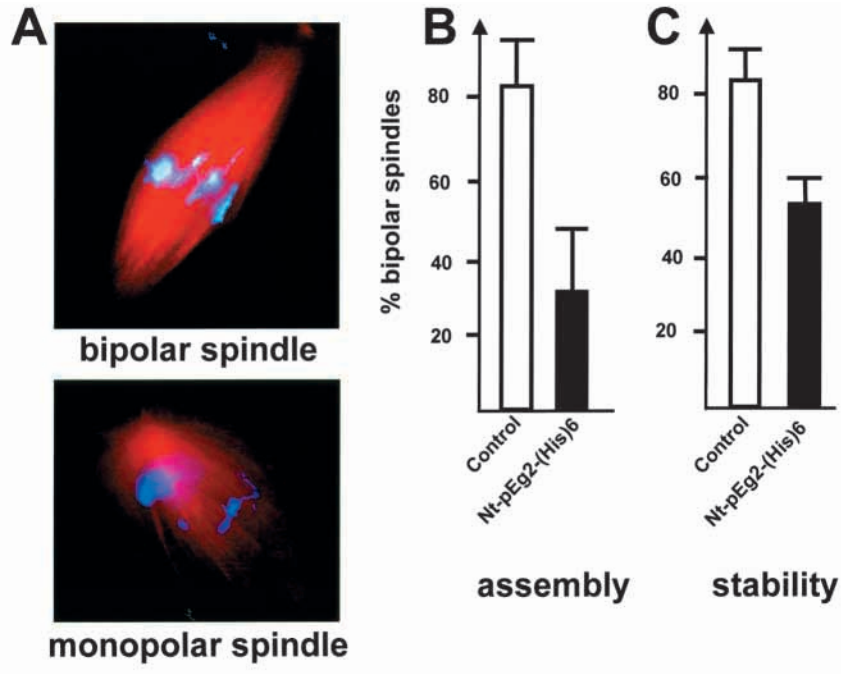

Fig. 7. The N-terminal domain of pEg2 inhibits bipolar spindle assembly and destabilises previously assembled bipolar spindles in *Xenopus* egg extract. The bipolar mitotic spindle assembly assay was performed as previously described (Roghi et al., 1998). After fixation, the spindles were scored under a fluorescence microscope. (A) Bipolar spindle (top) and monopolar spindle (bottom). The spindle incorporates rhodamine-labelled tubulin and appears in red. DNA is stained by Hoechst dye (blue). (B) 400 ng/µl (final concentration) of either the Nt-pEg2-(His)6 protein or a control pMAL peptide were added during spindle assembly. In the presence of Nt-pEg2-(His)6, only 34±18% (3 different experiments) of the spindles remained bipolar instead of 81±12% (4 different experiments) in the control. (C) Bipolar spindles previously assembled in *Xenopus* egg extract were incubated for 1 hour with 400 ng/µl of Nt-pEg2(His)6 protein, or with the control pMAL peptide at the same concentration. In the presence of Nt-pEg2-(His)6, 55±5% (3 different experiments) of the spindles remained bipolar instead of 82±8% in the control (4 different experiments).

DISCUSSION

AuroraA is a centrosome kinase and, although the function of the centrosome has been conserved throughout evolution (e.g. mitotic spindle microtubule nucleation), its structure is variable. The yeast centrosome 'Spindle Pole Body' is localised within the nuclear membrane and the spindle is assembled inside the nucleus (Winey and Byers, 1993; Snyder, 1994). The *Dictyostelium* centrosome 'Nucleus-Associated Body' has a layer-like structure without centrioles (Heath, 1981). The vertebrate centrosome is composed of two centrioles localised closed to the nucleus (Paintrand et al., 1992; Chretien et al., 1997). In order to fulfil its function, the auroraA centrosome kinase needs to interact with the centrosome structure. As the function of the kinase has been conserved throughout evolution a domain of the auroraA kinase must have been conserved (e.g. the catalytic domain) whereas another domain must have evolved together with the centrosome structure (e.g. the non-catalytic domain).

We have investigated the function of the non-catalytic domain of the *Xenopus* auroraA protein (pEg2) and found that this domain localises the kinase to the centrosomes in a microtubule-dependent manner. Using an in situ assay (Giet and Prigent, 1998), we estimated the affinity of the protein for the centrosome at the spindle poles. Whereas the pEg2-(His)6 protein is removed from the centrosome by 300 mM NaCl, only 150 mM NaCl is required to remove Nt-pEg2-(His)6 (data not shown), suggesting that the N-terminal domain has less affinity for the centrosome than the full-length kinase. In fact both the N-terminal domain and the catalytic domain of pEg2, when fused to the GFP, localise to centrosomes when transfected into *Xenopus* cells. However, again the efficiency of the N-terminal domain to localise to centrosomes is threefold higher. We think that the localisation mechanisms of the two domains are different. The N-terminal domain uses an active mechanism that needs microtubules, whereas the catalytic domain binds directly to the kinase substrates already located at the centrosome. The kinesin-related protein XlEg5, for instance, which is a substrate for pEg2, localises in vivo to mitotic centrosomes after pEg2, during cell cycle progression. The catalytic domain of the kinase associates with XlEg5 through the two-hybrid system (Giet et al., 1999) whereas the N-terminal domain does not bind to this motor protein.

One possible localisation mechanism for the full-length pEg2 is that the kinase might be targeted via its N-terminal domain to the centrosomes where it then finds and/or waits for its substrates. Functionally, this localisation mechanism would bring the kinase close to substrate with which it can interact. Once the catalytic domain binds to the substrate, the affinity

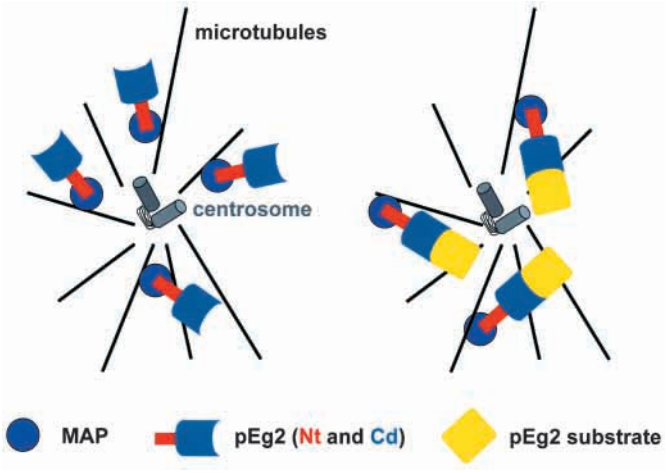

Fig. 8. Localisation mechanism of pEg2. The N-terminal domain (red) localises the kinase to the centrosomes (grey) through an interaction with a hypothetical protein (blue) that binds to microtubules (black), the localisation being microtubule-dependent. Then the kinase binds to its substrates (yellow), which stabilises the localisation so that it becomes microtubule-independent.

of pEg2 for the centrosome increases; that was observed in situ: 150 mM NaCl was required to release the Nt-pEg2-(His)6, versus 300 mM for the full-length kinase (Giet and Prigent, 1998).

Fig. 8 shows one possible mechanism for pEg2 localisation. In the absence of any substrate, pEg2 kinase would localise to the centrosomes via its N-terminal domain through interaction with a hypothetical microtubule binding protein. Then, when the substrate localises to the centrosome the kinase interacts with it. We suggest that the full-length kinase is targeted to the centrosomes via its Nt-domain and the localisation is stabilised by interaction with substrates like XlEg5. XlEg5 localisation in the centrosome is microtubule independent (data not shown).

Why and how the presence of microtubules is needed for the localisation of the N-terminal are still under investigation. We are, for instance, currently searching for proteins that target this N-terminal domain to the centrosome in a microtubule-dependent manner. Proteins such as dynein, which is a microtubule minus end directed motor, are good candidates for bringing the kinase to the centrosomes, as has been shown for NuMA (Gaglio et al., 1996; Walczak et al., 1998; Merdes et al., 2000). Recently, the *Xenopus* kinase auroraB (mid-body pEg2 paralogue) has been found associated with INCENP in egg extract (Adams et al., 2000). The authors demonstrated that in Hela cells, disruption of INCENP localisation leads to a loss of auroraB staining, indicating that the association is required for localisation of the kinase. It would be interesting to know if auroraB interacts with INCENP through its N-terminal domain since, like auroraA, auroraB binds to a kinesin-like protein during cytokinesis (Severson et al., 2000).

In human cells three different aurora kinases (pEg2 homologues) are localised on the mitotic apparatus: auroraA at the spindle poles and the centrosomes, auroraB at the midbody and auroraC at the centrosomes only in anaphase (Shindo et al., 1998; Terada et al., 1998; Kimura et al., 1999). Each of these three kinases has a conserved catalytic domain (80% identities between auroraB and auroraC), but a very different non-catalytic domain.

It will now be important to determine if the non-catalytic domains of the three human kinases are also localisation domains. The localisation mechanism of auroraB in *C elegans*, for instance, is quite complex, depending on three proteins, the chromosome passenger INCENP, the survivin-like protein Bir-1 and the mitotic kinesin-related protein Zen-4, with none of these appearing to be auroraB substrates (Adams et al., 2000; Severson et al., 2000; Speliotes et al., 2000).

Because mitosis is very short and extremely dynamic the mechanisms that determine protein localisation to the mitotic apparatus are predicted to result from transient association. The dissection of these localisation mechanisms will be of a great help to our understanding of chromosome segregation.

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