INTRODUCTION

The mitotic spindle orchestrates alignment of the chromosomes and proper segregation of the sister chromatids to ensure the correct repartition of genetic material during cell division. Spindle assembly and dynamics critically depend on microtubule-associated motors (Gelfand and Scholey, 1992; Hoyt, 1994; Barton and Goldstein, 1996; Walczak and Mitchison, 1996).

The bimC motors subfamily are slow plus-end-directed KRPs which function as bipolar homotetramers that cross-link parallel and antiparallel microtubules to bundle them or slide them apart (Cole et al., 1994; Gordon and Roof, 1999). They have been identified in a wide range of organisms: bimC in Aspergillus nidulans (Enos and Morris, 1990), Cin8 and Kip1 in Saccharomyces cerevisiae (Hoyt et al., 1992; Roof et al., 1992), Cut 7 in Schizosaccharomyces pombe (Hagan and Yanagida, 1990), Cut7 in Xenopus laevis (Enos and Morris, 1990), Ctn8 and Kip1 in Schizosaccharomyces pombe (Hagan and Yanagida, 1990), Eg5 in Xenopus laevis (Sawin et al., 1992), HsEg5 in Homo sapiens (Blangy et al., 1995), KLP61F in Drosophila melanogaster (Heck et al., 1993), TKRP110 and TKRP170 (Chui et al., 2000). They all localize to the mitotic spindle in a microtubule-dependent manner. Loss of function studies using genetic mutations, injections of antibodies in cells and depletion experiments in mitotic extracts always result in the formation of embryos with polyplody and multiastral blastomeres. Immunofluorescence analysis indicated that these defects did not arise from failure in either centrosome separation or bipolar spindle formation. Time-lapse observations showed rather that these perturbations in cell division resulted from abnormal anaphase and failure to complete cytokinesis. These phenotypes differ from the phenotype described following perturbation of the function of bimC family members in other organisms. Our study has thus uncovered roles for a bimC kinesin in late stages of cell division.

SUMMARY

We have isolated and characterized Boursin, a kinesin-related protein of the bimC family, from Paracentrotus lividus sea urchin eggs. Boursin is expressed at high levels in eggs and embryos during early cleavage stages. Boursin was found to be associated with different parts of the mitotic spindle from early prophase to telophase. Expression of a form of the protein predicted to act as a dominant negative mutant caused severe defects in cell division and resulted in the formation of embryos with polyplody and multiastral blastomeres. Immunofluorescence analysis indicated that these defects did not arise from failure in either centrosome separation or bipolar spindle formation. Time-lapse observations showed rather that these perturbations in cell division resulted from abnormal anaphase and failure to complete cytokinesis. These phenotypes differ from the phenotype described following perturbation of the function of bimC family members in other organisms. Our study has thus uncovered roles for a bimC kinesin in late stages of cell division.

Key words: Sea urchin embryo, Kinesin-related protein, bimC, Mitosis, Cytokinesis

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Additional roles for bimC proteins have been suggested and emphasized more recently. Studies in fungi, Drosophila and human cells suggest that the bimC motors are important for the maintenance of the centrosome-centrosome distance rather than centrosome separation (Hoyt et al., 1993; O’Connell et al., 1993; Sharp et al., 1999b; Mountain et al., 1999). In Drosophila, KLP61F is necessary during anaphase B and telophase as inhibition of KLP61F in the ncd null mutant (a minus end directed KRP), results in defects in microtubules organization within the spindle midbody and in a decrease in the relative distance of the daughter nuclei (Sharp et al., 1999b; Sharp et al., 2000). It has also been hypothesized that HsEg5 and Eg5 could play a role in cytokinesis according to their accumulation in the midbody during late telophase (Whitehead and Rattner, 1998; Giet et al., 1999). According to this hypothesis, bimC motors, like some other spindle KRPs, might play a role in cleavage stimulation at the end of mitosis.

The sea urchin embryo is a well defined system to study mitosis and cytokinesis. Injection of pan-kinesin antibodies have already suggested that KRPs are important players in these events (Wright et al., 1993). Recently, four novel sea urchin KRPs, Kinesin-C (Rogers et al., 1999), KRP180 (Rogers et al., 2000), KRP110 and KRP170 (Chui et al., 2000) have been identified and implicated in mitosis or cell division in sea urchin embryos. In an attempt to better understand the role of the bimC proteins and investigate their implication in cell division in sea urchin embryos, we have identified and characterized their homologue, ‘Boursin’, in Paracentrotus lividus eggs. The sequence of Boursin described here is 95% similar to that determined independently for KRP170 (Chui et
MATERIALS AND METHODS

Animals, gametes and embryos

Adults Paracentrotus lividus were collected in the bay of Villefranche-sur-mer. Animals were opened, sperm was kept ‘dry’ at 4°C and oocytes were obtained by soaking the female gonads in seawater followed by filtration through a 150 μm Nitex membrane. Eggs were fertilized in seawater containing 1 mM p-aminobenzoic acid and fertilization envelopes were removed by filtration through 70 μm Nitex. Zygotes were cultured at 18°C under gentle stirring.

Isolation of boursin cDNA

Minus strand cDNA was synthesized from blastula stage embryos total RNAs using the AMV reverse transcriptase (Amersham, Buckingham, UK) and oligo d(T) as a primer. A partial cDNA transcript was amplified by PCR using primers derived from the conserved regions FA YGQTG and NKPEVNQK of the motor head of bimC kinesin-like proteins (forward: 5¢-TTYGCNTA YGGNCARACNGG-3¢; reverse: 5¢-TTYTGRTTINACYTTCNGGYTTTRT-3¢). A single fragment corresponding to the expected length (800 bp) was amplified and cloned into Bluescript. This fragment was sequenced using the Amersham kit and found to be very similar to the expected bimC partial sequence. It was subcloned into the pBluescript II plasmid. This plasmid was transformed into E.coli, and the resulting colonies were screened for the presence of the expected fragment. A single positive clone was selected and sequenced. The sequence was submitted to GenBank and assigned the accession number. The sequence of the bimC cDNA was determined using dideoxy chain termination sequencing and the sequence was confirmed to be correct.

Plasmid constructions and in vitro mRNA synthesis

The sequence corresponding to the sea urchin bimC boursin was cloned into the CS2+ expression vector (Turner and Weintraub, 1994) using a two step protocol. First, a fragment of 394 bp containing the ATG codon was cloned into the Bluescript II plasmid using a forward primer containing an EcoRI restriction site (underlined) 5¢-CCTTGGAATTTCAAGGTCAAGGTGTTGAAAACCC-3¢ and a reverse primer containing BglII and XhoI restriction sites 5¢-CCAACTC-GAGAGATCCTGTTTCCATGAAAGATCGGG-3¢. This fragment was amplified and purified, digested by EcoRI and XhoI and cloned into CS2+ to obtain the plasmid CS2+394. Second, the full-length cDNA Boursin was digested by BamHI and DraI and cloned into CS2+394 previously linearized by BglII, blunted and digested by XhoI.

The construction encoding the rigor-type mutant Boursin was performed in two steps as described above but a point mutation was introduced by replacement of two bases leading to the substitution of threonine 304 by an asparagine. The forward primer used for the PCR was the same as above and the reverse primer containing the mutation (bold) was 5¢-CCAACTGCAGAGATCCTGTTTCCATGAAAGAGA-

TCAGGCTTCTGTCTCCTCCATCTCCATGAAATCTTCTTCTGTTACCCAG-3¢. CS2+ constructs were linearized with NotI and used for in vitro synthesis of capped mRNAs with a mMessage mMachine™ kit (Ambion) using SP6 polymerase. The quality of synthetic mRNAs was analyzed by gel electrophoresis. RNA was resuspended at 2 μg/μl in 10% glycerol-DEPC-treated water.

Microinjections

Microinjections were carried out according to MacMahon et al. (1985). Briefly, dejellied oocytes were lined up on a Petri dish cover coated with 0.25% protease and injected in their cytoplasm with about 2 pl of mRNA or buffer solution using a continuous flow microinjector apparatus. Injected oocytes were fertilized in situ and cultured at 18°C in fresh 0.22 μm filtered seawater.

Antibody production and purification

The tail coding region (bp 2562 to bp 3354), which is the most specific part of Boursin, was chosen to produce polyclonal antibodies. This region was amplified by PCR with the pfu polymerase (Stratagene) using forward and reverse primers containing an EcoRI and a NotI site, respectively (5¢-GGTTGGAATTCTGACCCACCCACACC-3¢; 5¢-GGTTTGCCGCCACTTTAGCGAGAATGTCCAGAGC-3¢). The amplified fragment was purified, digested by EcoRI and NotI and cloned into vector pET 30b+ (Novagen) in order to produce a tail-poly (His) fusion protein. Induction of protein synthesis was performed by addition of 1 mM IPTG followed by an overnight incubation at room temperature. The soluble fusion protein was purified as recommended by Novagen, dialyzed against PBS and used to immunize two rabbits by 3 subcutaneous injections. The anti-tail polyclonal antibodies produced were purified on an affinity column made of the antigen covalently bound to Affigel-10 (Bio-Rad). After an extensive wash with MOPS buffer, the antibodies were eluted with 10 volumes of 2 M glycine, pH 2.3, and then dialyzed overnight against 2 liters of PBS containing 0.5 M KCl and 30% glycerol. They were then stored at −80°C. The affinity-purified antibodies were >95% pure IgG as judged by Coomassie blue staining of SDS-PAGE gels. The final antibody concentration was 850 μg/ml.

Preparation of sea urchin extracts and western blot analysis

At the desired stages of development, embryos were harvested and treated according to the method of Gan and Klein (1993). Embryos were washed with 0.55 M NaCl and then 1 M glucose. Pelleted embryos were resuspended and lysed in 10 volumes of a buffer containing 25 mM Hepes pH 7.8, 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1 mM spermidine and protease inhibitors, supplemented with 1/10 volume of 4 M (NH4)2SO4 and incubated 30 minutes at 4°C under agitation. After centrifugation of the lysate (1200 g, 15 minutes, 4°C), the pellet was resuspended in a minimal volume of a buffer containing 25 mM Hepes, pH 7.9, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT and protease inhibitors and dialyzed overnight at 4°C against the same buffer. Protein extracts were denatured for 5 minutes at 95°C in SDS loading buffer (Laemmli, 1970). Proteins were then resolved by SDS-PAGE prior to be transferred onto nitrocellulose membranes (Schleicher and Schuell) for 1 hour at 100 V using a transfer cell system (Bio-Rad). The membranes were pre-incubated for 1.5 hours in a blocking solution (PBS containing 0.1% Tween-20 and 3% dry non-fat milk) and then incubated overnight at 4°C in the same solution containing the purified polyclonal Boursin antibody (final concentration 400 ng/ml). The membranes were then washed in PBS containing 0.1% Tween-20 and 3% dry non-fat milk and then incubated overnight at 4°C in the same solution containing the affinity-purified antibodies. The membranes were then washed in PBS containing 0.1% Tween-20 before being incubated for 1 hour at room temperature with an anti-rabbit secondary antibody covalently coupled to horseradish peroxidase (Amersham) diluted at 1/7500 in the washing buffer. After 3 further washes, the immunological reaction was developed using the ECL system (Amersham).
Immunofluorescence staining

Paracentrotus lividus embryos were collected at different time points and fixed according to McNally (McNally, 1996). Briefly, embryos were rinsed in PMEG buffer (100 mM Pipes-KOH, 1 mM MgSO4, 1 mM EGTA, 10% glycerol, pH 6.8) and adhered to polysyline-coated slides prior to immersion overnight in a 90% methanol-50 mM EGTA solution at −20°C. Immunofluorescence staining was performed at room temperature. Fixed embryos were first re-hydrated in PBT buffer (PBS, 4% BSA, 0.05% Triton X-100) for 10 minutes and then incubated in the same buffer containing the primary antibodies. After an hour, embryos were rinsed several times and incubated with the secondary fluorescent antibodies diluted in PBT. DNA was stained with Hoechst 33258 (0.2 μg/ml in PBT) (Sigma). After further washes, the samples were mounted between slides and coverslips in a drop of Citifluor mounting medium (Kentucky, UK). Embryos were observed using a Zeiss inverted microscope, with a ×40 oil immersion objective. All images were processed using Metamorph imaging system software (Universal Imaging Corporation).

The primary antibodies used were: YL1/2 anti-alpha tyrosylated tubulin (Sigma, used at 1:1000); anti-stalk Boursin (used at 1:50), and anti-katanin (used at 1/10, generous gift from F. McNally, University of California, Davis).

The secondary antibodies used were: Texas-Red-conjugated anti-rat IgG (Jackson Research, used at 1:250), CY2-conjugated anti-rabbit IgG (Jackson Research, used at 1:200), FITC-conjugated anti-mouse IgG (Zymed, used at 1:200).

Time-lapse observations

Embryos were collected immediately after injection and fertilization and allowed to develop in a small Petri dish coated with GF (0.1% gelatin and 0.1% formaldehyde). At the 4- or 8-cell stage, embryos were immersed for 10 minutes in calcium-free seawater to allow blastomere dissociation, containing 0.5 μg/ml Hoechst 33258. After several washes in calcium-free seawater, dissociated blastomeres were collected and placed into little chambers made of GF-coated coverslips surrounded by Plexiglas walls. They were observed under an inverted Zeiss microscope using transmitted and u.v. lights for at least 2.5 hours. Images were recorded every 2 minutes using a Metamorph imaging program (Universal Imaging Corporation).

RESULTS

Boursin, a bimC kinesin related protein in sea urchin eggs

In order to study the role of bimC proteins in cell division, we looked for the sea urchin homologue in Paracentrotus lividus. A DNA fragment encoding part of bimC homologue was amplified by RT-PCR using degenerate oligonucleotides corresponding to conserved regions of bimC family members. This fragment was used as a probe to screen a cDNA library prepared from unfertilized sea urchin eggs RNAs. Five positive clones were isolated and further purified. The longest cDNA was sequenced on both strands. This cDNA was 3841 nucleotides long with 109 nucleotides 5′ flanking sequence and 489 nucleotides 3′ untranslated region. It contained a unique long open reading frame, which started at the ATG 109 and ended at the stop codon 3352, predicted to encode a 1081 amino acid bimC protein with an estimated molecular mass of 123 kDa. Like other bimC proteins, the sea urchin protein ‘Boursin’ (for BimC d’Oursin, french name for sea urchin) is composed of three distinct regions (Fig. 1A). The N-terminal portion of the molecule (357 amino acids) corresponded to the globular kinesin motor head. It contains the ATP binding site found in all bimC family members (97FAYGQTGTGK106), and the consensus motifs found in motor domain of KRPs (156EXYYXXXXXDLL167, 226SSRSRSH230 and 257VDLAGS-E263). The central 460 amino acid (residues 358-817) is predicted to adopt a coiled-coiled conformation and the C-terminal tail (residues 818-1081) contains the ‘bimC box’ (935PTGTTP940), a consensus sequence with a potential phosphorylation site for the mitotic kinase p34cdc2 (Blangy et al., 1995; Sawin and Mitchison, 1995). Sequence alignments of Boursin with other bimC proteins revealed a high degree of conservation in the motor domain. The sequence of Boursin displays 72% identity with the Xenopus Eg5 in its putative motor domain and 28% in the stalk domain. The tail is specific to each species, and the Boursin tail shares only the consensus bimC box with the other members of the bimC family.

Boursin protein accumulate during early cleavage stages

The presence of Boursin protein during various stages of sea urchin development was analyzed using a specific antibody raised against the carboxy-terminal tail domain of Boursin.

![Fig. 1. (A) Map of domain organization predicted from Boursin sequence. ATP binding site of bimC family members, consensus sequences of kinesin superfamily members, bimC box. The sequence data for Boursin is available from EMBL database Library under accession number AJ293506. Recombinant poly histidine Boursin Stalk/Tail domain fusion protein used to raise anti-Boursin antibody. (B) Expression of Boursin protein during development. Western blot analysis of total protein extract prepared from embryos at different stages of development. The membrane was incubated with a specific affinity purified anti-Boursin antibody. UF: unfertilized eggs; 16: 16-cell stage; 16: 16-cell stage; 32: 32-cell stage; B6-B7-B11: blastula stage at 6, 7 and 11 hours post-fertilization, respectively; MB: mesenchyme blastula; G: gastrula. Numbers indicate molecular mass markers in kDa.](Image 239x166 to 568x398)
This antibody recognized a doublet at ~130 kDa, a size close to the value calculated for the (Boursin protein (Fig. 1B, arrows). The presence of a doublet may reflect different degrees of phosphorylation of the protein or may correspond to the presence of two slightly different proteins as has been suggested for Eg5 in Xenopus and for TKRP125 in tobacco cells (Houliston et al., 1994; Asada et al., 1997). Boursin was first detected in unfertilized eggs, its amount increased gradually during the period of rapid cleavages peaking at the blastula stage (11 hours after fertilization) and thereafter decreased to an undetectable level at the mesenchyme blastula stage (18 hours post-fertilization). This temporal expression is consistent with a requirement of Boursin in cell division during period of intense cleavages.

**Boursin localizes to the mitotic spindle**

The subcellular localization of Boursin during the first cell cycle was analyzed by indirect immunofluorescence using affinity purified anti-Boursin antibody (Fig. 2f-j) and anti-tubulin antibodies to determine mitotic stages (Fig. 2a-e).

In unfertilized interphasic eggs, only diffuse cytoplasmic stainings of Boursin and tubulin were observed (data not shown). 10 minutes post-fertilization the sperm nucleus, pushed by the growing sperm aster (Fig. 2a, arrow), migrated towards the female nucleus. Boursin became localized at this stage (Fig. 2f, arrow) and was associated with the sperm aster microtubules. During late prophase, (Fig. 2b) it was possible to detect centrosomal staining on the nascent asters (Fig. 2g). At metaphase (Fig. 2c), Boursin was found along the spindle microtubules in addition to the asters (Fig. 2h). During anaphase, the asters enlarged considerably and the spindle elongated (Fig. 2d). Boursin was highly concentrated between the chromosomes and the poles and, to a lesser extent, on the central spindle and on astral microtubules (Fig. 2i). As telophase progressed (Fig. 2e), the staining of Boursin intensified in the midbody region (Fig. 2j, arrowhead) and the protein was diffuse in the cytoplasm as well.

**Expression of rigor-type mutant Boursin in embryos causes polyploid and multiastral cells**

In order to understand the function of Boursin in sea urchin eggs, we constructed a mutant version of the protein. A single mutation of Threonine 355 to Asparagine in the ATP loop of the motor head of kinesin proteins leads to the formation of proteins that show rigor-type binding to microtubules due to their inability to hydrolyze ATP (Nakata and Hirokawa, 1995; Blangy et al., 1998). Such a mutant protein has been tested in HeLa cells for HsEg5 and the protein behaved as a dominant negative (Blangy et al., 1998). A synthetic mRNA predicted to encode an equivalent rigor-type mutant form of Boursin was made and injected into sea urchin eggs prior to fertilization. Embryos were then cultured until the 256-cell stage (6 hours post-fertilization), fixed, stained with anti-tubulin (Fig. 3) or anti-Boursin (Fig. 4) antibodies and observed by immunofluorescence microscopy.

Embryos injected with 10% glycerol or with mRNA encoding wild-type Boursin at 2 μg/ml (n >400) did not show any perturbations in development (Fig. 3a,d). They contained ordered mitotic and interphasic array of microtubules (Fig. 3a) and Boursin associated with microtubules, spindle poles and the central spindle (Fig. 4a), like during first mitosis. On the
contrary, 85% of embryos injected with mRNA encoding mutant Boursin were severely affected ($n>400$). Blastomeres of these embryos were fewer in number than blastomeres of control embryos (Figs 3 and 4b,c,e,f). Moreover, they were of irregular sizes and often bigger than blastomeres of control embryos. Staining of tubulin and DNA revealed that the affected blastomeres were multiastral and multinucleated (Fig. 3b-c and e-f). Microtubules were organized as asters distributed throughout the cells (Fig. 3c) or as disorganized arrays (Fig. 3b, arrow). Chromatin was present as many masses of various sizes (Fig. 3e, arrow) or as a single mass clumped in the middle of the cell (Fig. 3f, arrowhead). When associated with asters, DNA was condensed (Fig. 3F) and was decondensed when associated with interphase-like arrays of microtubules (Fig. 3e, arrow). Staining of Boursin in injected embryos revealed that Boursin was always associated with the disorganized microtubule structures or with the well-defined asters (Fig. 4b, arrowhead and arrow, respectively). The phenotypes observed were heterogeneous among different blastomeres within a single embryo. This was probably due to unequal partitioning of the injected mRNAs after each cell division.

To detect the earliest manifestation of the phenotype caused by the expression of a rigor-type form of Boursin, embryos were fixed between 4 and 6 hours after fertilization, and stained

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**Fig. 3.** Effect of a rigor-type mutant Boursin on sea urchin egg development. Eggs were injected with Boursin mutant encoding mRNA, fixed 6 hours after fertilization and prepared for anti-tubulin immunofluorescence. Double staining of tubulin and Boursin in mutant-injected embryos was not possible, probably because the large amount of recombinant protein bound to microtubules and masked the antigenic sites normally detectable by the anti-tubulin antibody. (a-c) Tubulin (YL 1/2); (d-f) DNA (Hoechst). Control injected embryo (a) had reached the 256-cell stage. Note that blastomeres divide asynchronously such that mitotic spindles and interphasic arrays are present in a single embryo. Injected embryos (b and c) contained various number of blastomeres of different sizes. Blastomeres were multinucleated (c, arrow) or had a single nucleus bigger than in controls (f, arrowhead arrowhead). Embryos displayed a disorganized array of microtubules (b, arrow) or are multiastral (c, arrowhead). Bars: 12 μm (a,b,d,e); 30 μm (c and f).

**Fig. 4.** Effect of a Boursin rigor-type mRNA injection on sea urchin egg development. Eggs were injected with the mRNA encoding Boursin mutant, fixed 4 hours post-fertilization (64 cells) and prepared for anti-Boursin labeling. (a-c) anti-Boursin; (d-f) DNA (Hoechst). In control embryos, Boursin was located to the mitotic spindle, highly concentrated at the poles and less abundant on the central spindle (a). In injected embryos, Boursin was associated with a disorganized array of microtubules (b, arrowhead), with asters in multiastral blastomeres (b, arrow) or with spindles (c, arrows indicate blastomeres containing two parallel spindles). Bar, 12 μm.
with anti-Boursin antibody as above when no phenotype could be detected by bright field microscopy (Fig. 4b,c). The earliest defects observed were the occurrence of double metaphase spindles lying parallel and sometimes interconnected within a single cell (Fig. 4c arrows). In such cells, Hoechst staining showed two distinct or continuous sets of condensed chromosomes aligned in the equatorial plane (Fig. 4f, arrows). In general, the spindle poles were slightly less focused and the spindle less well organized than in controls. These results show that embryos injected with the mutated Boursin mRNA were able to form bipolar spindles and suggest that the multinucleated cell phenotype resulted rather from defects in late mitosis and/or in cleavage.

Mutant Boursin protein does not prevent centrosome separation

In various organisms, inhibition of bimC motor functions leads to the formation of monopolar spindles with unseparated centrosomes (for review, Kashina et al., 1997) or sometimes monoastral bipolar spindle (Wilson et al., 1997). We never observed monopolar spindle in sea urchin embryos expressing the Boursin construct, but consistently observed multiastral or bipolar spindles in single blastomeres. In order to determine whether these asters were formed from truly separated centrosomes, injected embryos were stained with an antibody directed specifically against katanin, a protein known to be concentrated at the centrosome in the sea urchin Strongylocentrotus purpuratus (McNally et al., 1996). In control Paracentrotus lividus embryos, anti-katanin antibodies stained centrosomes brightly from prophase (not shown) to anaphase (Fig. 5a and c) and, although much more weakly, microtubules. At anaphase, the staining was large and adopted a halo shape (Fig. 5b). In mutant-injected embryos, centrosomal and microtubule-associated stainings were also observed (Fig. 5e). In cells with several metaphase spindles (Fig. 5d,f), a single katanin staining spot was present at each pole of each spindle (Fig. 5e, arrow). These spots were in general larger than those seen in control embryos but were never found as a doublet. These results indicate that, although the spindle poles in these cells were slightly larger than in control, centrosomes had separated correctly during bipolar spindle formation.

Boursin rigor-type protein causes abnormal anaphase and abortive cytokinesis

To understand the origin of the multi-spindle and multi-astral phenotypes, time-lapse microscopy recordings were carried out. Embryos injected with mRNAs encoding the mutant Boursin or with 10% glycerol (n>300 for each) were allowed to develop up to the 4 or 8-cell stage. In order to facilitate observations, the embryos were dissociated in calcium free seawater and the separated blastomeres were mounted in small chambers. Progression through the cell cycle was monitored by bright field microscopy and images were recorded every 2 minutes. In these conditions, blastomeres from glycerol injected embryos continued to develop normally with a typical cell cycle duration of 45 minutes. On Fig. 6A, the 4 blastomeres observed at the 4-cell stage proceeded through four successive rounds of mitoses (compare first to last panel) and asymmetric divisions of the 8-16 cell transition occurred normally to form the micromeres (Fig. 6A, 0h 56’). In contrast, a striking perturbation of cytokinesis was observed in most cells derived from embryos injected with mRNA encoding mutant Boursin (85%, n>100). On Fig. 6B, the behavior of four blastomeres isolated from an 8-cell stage embryo (small) and two isolated from a 4-cell stage embryo (big) is shown. All these blastomeres started to cleave, formed a well-defined cleavage furrow (see for example Fig. 6B, 0h 36’ and 0h44’, arrows), but did not complete their division and separation of the daughter cells rarely occurred. In the first panel, the arrow indicates a pair of sister blastomeres which have apparently completed cytokinesis. In fact, 30-40 minutes later, the cleavage furrow had regressed resulting in the formation of a single blastomere (Fig. 6B, 0h 50’). Other examples of apparent cleavage furrows which are later resorbed are indicated by the arrows in panels 0h 36’, 0h 44’ and 1h 10’. Some blastomeres undertook abortive cleavage attempts in successive mitoses. In these cases, several cleavage furrows could be observed within the cell (1h 43’, arrow), probably reflecting the presence of multipolar mitotic spindles.

In order to further characterize the phenotype observed, fluorescence microscopy observations of blastomeres isolated from injected embryos and in which the DNA has been stained with the vital dye Hoechst 33342 were carried out. In blastomeres from glycerol-injected embryos, interphase DNA (Fig. 7A, 0h 00’), and mitotic figures corresponding to metaphase (Fig. 7A, 0h 32’) and anaphase (Fig. 7A, 0h 41’ to 0h 47’) were easily distinguishable. In blastomeres from embryos expressing the mutant protein (80%, n>50), decondensed DNA and metaphase plates could be observed (Fig. 7B 0h 00’ and 0h 37’) but anaphase figures were less well defined (Fig. 7B, 0h 43’ to 0h 52’). Anaphase A seemed to be initiated normally as sister chromatids started to separate (Fig. 7B, 0h 43’) but did not complete as the separation was clearly not as well defined as in control blastomeres (compare Fig. 7A, 0h 41’ and 0h 44’ to Fig. 7B, 0h 46’ and 0h 49’). Although chromatids remained very close, the subsequent initiation of cytokinesis was not perturbed (Fig. 7B, 0h 52’) and the cleavage furrow seemed to ingress normally. At telophase, it has to be noticed that the two newly formed nuclei of the sister cells were very close one to another and were located near the cleavage plane (Fig. 7B, 0h 58’), in contrast to control blastomeres in which they were centered in each daughter cells (Fig. 7A). When focusing through the blastomeres, we observed that the two nuclei often remained connected (Fig. 7B, 1h 43’). Daughter cells were transiently formed and never completely separated. Thus, 45 minutes after its initiation, the cleavage furrow started to regress resulting in the formation of a unique large blastomere (Fig. 7B, 2h 00 to 2h 28’). In this abnormal blastomere, the DNA began to condense in several masses instead of two nuclei, as the cell was entering a new cycle of division (Fig. 7B, 2h 00 to 2h 16’). All these observations provide evidences that the Boursin kinesin is implicated in anaphase and in the completion of cytokinesis.

DISCUSSION

Boursin, a kinesin-related protein of the bimC family in sea urchin embryos

We have identified a sea urchin egg bimC homologue, ‘Boursin’, and analyzed its role during mitosis. Like all the
bimC family members, and like KRP170 recently identified in the sea urchin L. pictus (Chui et al., 2000). The sequence of Boursin is conserved in its motor domain and species-specific in its tail region outside of the consensus sequence of the bimC box. Boursin transcripts (data not shown) and protein were very abundant during the cleavage period and decreased markedly thereafter (Fig. 1B), consistent with a role for the protein during cell division. The subcellular localization of Boursin with specific antibodies showed that Boursin was associated with the mitotic spindle (Fig. 2) in a way similar to its homologues from the sea urchin L. pictus (Chui et al., 2000) and from other species. In order to analyze the function of the endogenous Boursin in vivo in sea urchin embryos, we constructed a mRNA encoding a rigor-type version of the protein by mutation in its ATP binding domain. Mutant Boursin protein behaved as a dominant negative protein and, considering its rigor-type characteristic, we cannot rule out the possibility that the phenotype we observed is due to a non specific effect linked to the inability of other microtubule associated proteins to reach and bind microtubules. The expression of the mutant Boursin caused a striking phenotype characterized by the presence of polyploid blastomeres containing multiple asters or two apparently separated or interconnected bipolar spindles (Figs 3, 4). Further analyses showed that anaphase was disturbed with only partial separation of sister chromatids and that cytokinesis completion was defective (Figs 6 and 7). The ultimate separation of the two daughter cells rarely occurred even though the initiation of the furrowing did not seem to be affected and timing of cytokinesis never appeared to be delayed.

The results presented here are different from what has been previously seen in HeLa cells after expression of a full length rigor-type HsEg5 (Blangy et al., 1998) or for mutants of the bimC protein in various species. Perturbation in the function of bimC proteins in these systems also leads to the formation of polyploid cells, but this phenotype is always due to the formation of monopolar spindles. Formation of these monopolar spindles was explained in terms of early mitotic defects, i.e. failure in centrosome separation during prophase (for review, Kashina et al., 1997) or bipolar spindle collapse at metaphase (Sharp et al., 1999b). It is possible that spindle collapse occurred in sea urchin like in other species but this event is certainly rare as we never detected any monopolar spindle by anti-tubulin staining.

**Boursin is essential for anaphase**

Our immunostaining results showed that Boursin was concentrated at the poles but was also present, albeit less abundant, in the central spindle as was observed for KRP170 (Chui et al., 2000). Previous studies have shown that bimC proteins are active as tetramers. Like some other bimC family members, Boursin could cross-link interdigitated microtubules of the central spindle and slide them apart when moving towards their plus ends (Saunders et al., 1997; Walczack et al., 1998; Sharp et al., 1999a). The rigor-type mutant Boursin with its incapacity to move along microtubules may inhibit anaphase spindle elongation leading to partial separation of the chromatids. In this case, it is possible that the prevention of the migration of the daughter nuclei could compromise the reassembly of the nuclear envelopes at telophase resulting in the formation of two or more aberrant nuclei. These aberrant nuclei could also result from chromosome missegregation. In Fig. 7 (panel 1h 43’), masses of chromosomes do not seem very well individualized and remain linked together. Studies in Drosophila embryos have shown that KLP61F is involved in anaphase B in the organization of midzone microtubules and in the maintenance of relative distance between the daughter nuclei (Sharp et al., 1999b) but chromosomal missegregation was never observed. Measurements of the distance between the spindle poles and the velocity of chromatic migration will be necessary to further characterize which step of anaphase is perturbed in injected embryos.

**Boursin rigor-type leads to abortive cytokinesis completion**

Cytokinesis completion is defective in embryos expressing the rigor-type mutant Boursin. To explain this observation, we favor a model in which cytokinesis disruption is a consequence of the anaphase defect. The incomplete separation of the two daughter nuclei or chromosome missegregation could prevent cytokinesis completion without affecting cytokinesis initiation (Fig. 7B 0h 58’ to 2h 28’). It has already been shown that severe chromosome segregation defects can in turn inhibit cytokinesis giving rise to truly multinucleated cells (Schultz and Onfelt, 1994).

An alternative model could implicate Boursin in the structure of the midzone. In many organisms, midzone microtubules are responsible for the positioning of the cleavage furrow and the completion of cytokinesis (Wheatley and Wang, 1996; Giansanti et al., 1998; Gatti et al., 2000; for reviews, Oegema and Mitchison, 1997; Wheatley, 1999). Previous studies have reported that disruption of the function of KRPs implicated in the structure of the midzone, like KRP170 subfamily, MKLP1 in mammalian cells (Nislow et al., 1992), Pavarotti in Drosophila (Adams et al., 1998) and Zen4 in C. elegans (Raich et al., 1998; Powers et al., 1998) or the chromokinesin KLP3A in Drosophila (Williams et al., 1995), provoke a failure in cytokinesis or a furrow regression. Boursin could directly cross-link antiparallel microtubules to organize the midzone as KLP61F does in Drosophila embryos (Sharp et al., 1999a).

Further experiments are required to determine the possible implication of the midzone in sea urchin cytokinesis and the role Boursin could play in midzone formation by analysing the organisation of the microtubules in this region in injected embryos.

Finally, Boursin accumulates in the midbody at the end of telophase and could be responsible for the localization of some components crucial for the completion of cleavage. In Xenopus eggs and HeLa cells, a possible role for Eg5 and HsEg5 during cytokinesis has also been proposed based on their localization at the midbody (Giet et al., 1999; Uzbekov, 1999; Whitehead and Rattner, 1998). This has been confirmed in Drosophila germ cells in which KLP61F is recruited to the midbody at the end of mitosis and accumulates at the fuses. The analysis of KLP61F mutants indicates that this KRP could be necessary to recruit fusome material such as the adducin-like protein, to the midbody at telophase (Wilson, 1999). The direct association of bimC KRPs with other proteins has been firmly established only in Xenopus in the case of the mitotic aurora-like protein kinase Eg2. Putative partners involved later in the cell cycle are yet to be identified.
Fig. 5. Localization of katanin in control and injected embryos. Embryos were fixed 4 hours post-fertilization (64 cells) and labeled for tubulin (a,d), katanin (b,e) and DNA (c,f). In control embryos (a,b,c), katanin was associated with the centrosomes in a ‘halo’. In injected embryos (d,e,f), katanin was associated with each spindle pole but this staining was more widely spread than in controls. Bar, 12 μm.

Fig. 6. Cytokinesis completion is abortive in embryos expressing Boursin mutant. Blastomeres from embryos injected with 10% glycerol or with mRNA were dissociated at the 4 or 8-cell stage and recorded under transmitted light. (A) Control blastomeres cleaved regularly every 45 minutes. At the 16-cell stage, the asymmetric division occurred and micromeres were formed (0h 56'). (B) Blastomeres from embryos expressing Boursin mutant. Most of blastomeres began cytokinesis, but after a few minutes, the cleavage furrow regressed (arrows). Bar, 12 μm.
Is Boursin involved in early mitosis?

The phenotype observed in embryos expressing the mutant Boursin does not preclude a role for Boursin during early steps of mitosis. In a recent work (Chui et al., 2000) the effect of anti-KRP170 antibody injection in *L. pictus* was studied and a major block in prophase and also a block in anaphase were observed, implicating KRP170 in mitosis and cell division. In *P. lividus* sea urchin eggs, proteins with a partially redundant function might then be able to compensate for loss of Boursin function during centrosome separation. Alternatively, the balance between endogenous Boursin and the mutant form may be critical: the level of the mutant protein could be insufficient to totally block

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**Fig. 7.** Rigor-type Boursin induces anaphase defects. Same conditions as in Fig. 6. Blastomeres from 8 cell embryos are shown. Time-lapse recordings were performed under transmission and epifluorescent illumination. (A) Control blastomeres complete their division within 45 minutes. 0h 32’: metaphase, 0h 41’ to 0h 44: anaphase, 0h 47’: cleavage. (B) In blastomeres of injected embryos, anaphase was incomplete (0h 49’), chromatids did not separate properly (2h 00’), the cleavage furrow regressed and the formation of new nuclei was perturbed (2h 16, 2h 28’). Bar, 12 μm.
early function in mitosis but sufficient to alter late events of cell division. It may be one of these possibilities that allowed us to distinguish a role for Boursin in late mitosis and in cytokinesis. Our immunolocalization data showed a clear association of Boursin with the mitotic spindle, mainly around the centrosomes and between the chromosomes and the poles (Figs 2 and 4a). This association with the spindle is common to all bimC motors for which involvement in bipolar mitotic spindle formation has been demonstrated. Even though we were not able to block early mitotic events, either by antibody microinjection (data not shown) or by a dominant negative approach, the phenotype obtained after expression of a rigor-type Boursin suggests that this protein does play a role during early mitosis. We found that bipolar spindles had less focused poles compared to controls (Fig. 5). In addition, and probably as a consequence of the diffuse pole structure, the spindle microtubules seemed to be less well aligned, but it was difficult to estimate if they were as abundant as in controls. In HeLa cells, the spindle poles become enlarged when HsEg5 function is blocked by antibody injections (Gaglio et al., 1996; Whitehead and Rattner, 1998). These observations have led to a model which stipulates that bimC proteins might be anchored in a pericentriolar matrix and could concentrate the minus end of microtubules at the poles by their plus end directed motor activity (Gaglio et al., 1996; Walczak and Mitchison, 1996).

The present work demonstrates that perturbation of a bimC kinesin affects late steps of mitosis and cytokinesis without formation of monopolar spindles. Until recently, it was always proposed that bimC proteins were implicated in centrosome separation. However, the finding in Drosophila embryos that KLP61F is responsible for the maintenance of centrosome distance and that this protein also plays a role during anaphase B and telophase have led Sharp et al. (1999b) to propose the complete re-evaluation of the function of the bimC proteins in all organisms. Our results in sea urchin lend support for this idea and we believe that all bimC proteins share common functions but also exhibit some differences. Sea urchin embryos have already shown differences from other organisms in the establishment and the regulation of the cleavage plane (Oegema and Mitchison 1997) and the involvement of Boursin, in anaphase and cytokinesis completion may reflect species differences in the exact roles that the bimC proteins might play. The bimC family of KRP s has been established on the basis of the high sequence homology between its members. This homology is restricted to the head and the stalk domains of the proteins. The tail region is very different and this variability could be at the origin of some fluctuation of function from one organism to the other. It would thus be interesting to see if the late roles played by Boursin are shared by the homologues from other species.

We thank Drs Janet Chenevert, Evelyn Houlston and Philippe Huitorel for their helpful comments on the manuscript. We acknowledge the financial support of the Centre National de la Recherche Scientifique (CNRS, France) and the Association pour la Recherche contre le Cancer (ARC) to G.P., and the Ministère de l’Education Nationale France) for a Thesis fellowship to I.T.

REFERENCES


McMahon, A. P., Flytzanis, C. N., Hough-Evans, B. R., Katula, K. S.,
Sea urchin KRPs in cell division


