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Cybip, a starfish cyclin B-binding protein, is involved in meiotic M-phase exit

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Abstract

We designed a screen to identify starfish oocyte proteins able to bind monomeric cyclin B by affinity chromatography on a cyclin B splice variant displaying low affinity for cdc2. We identified a 15 kDa protein previously described as a cdk-binding protein [Biochim. Biophys. Acta Mol. Cell Res. 1589 (2002) 219–231]. Cybip is encoded by a single polymorphic gene and the native protein is matured by cleaving a signal peptide. We firmly establish the fact that it is a true cyclin B-binding protein, since the recombinant protein binds recombinant cyclin B in absence of any cdk. Finally, we show that the microinjection of GST-cybip, and of anti-cybip antibody, in maturing starfish oocytes, inhibits H1 kinase and MPF inactivation, and first polar body emission.

Following the demonstration that MPF, purified from starfish maturing oocytes, was a heterodimer composed of one molecule of Cdc2 and one molecule of cyclin B [1], cyclins have been shown to be the essential regulatory subunits of cyclin-dependent kinases (CDKs). In starfish and in Xenopus prophase-arrested oocytes, MPF is present as an inactive pre-MPF, a heterodimer in which the Tyrosine 15 and the Threonine 14 of p34cdc2 are maintained phosphorylated, as the result of a balance between sustained Myt1 kinase activity and low Cdc25 phosphatase activity [2,3]. Strikingly in starfish, the activation of the prophase pool of pre-MPF is sufficient to allow progression through the whole first meiotic cell cycle, even in the absence of newly synthesized cyclin B [4]. Even so, the concentration of cyclin B is generally considered as a limiting factor, while p34cdc2 is present in excess, both in somatic and embryonic cells: the whole pool of cyclin B is thought to be complexed with Cdc2 in pre-MPF or MPF, while part of p34cdc2 molecules remain monomeric, some of them being able to undergo activating phosphorylation on Threonine 161 [5]. But even T161-phosphorylated Cdc2 molecules are not active as H1-kinase in the absence of associated cyclin B. The significance of the unbalance between concentrations of cyclin B and Cdc2, and also of the co-existence of differentially regulated Cdc2 molecules remains largely elusive.

Recently, we have identified in sea urchin a splice variant of cyclin B mRNA (cyclin Bsv), of which the 8th intron is not cleaved [6]. The result in the protein sequence is the complete removal of the C-terminal alpha helix, which is replaced by the intron-encoded sequence. Similar variants, found also in several additional models including starfish as we report in the present paper, are characterized by a greatly reduced affinity for p34cdc2 in vivo. While they are unable to compete with normal cyclin B for binding to Cdc2, the over-expressed variant impedes H1 kinase activation and severely delays entry into M phase [6]. This suggested that it could sequester an activator of the pre-MPF, able to bind even monomeric cyclin B.

In an attempt to identify ligands of monomeric cyclin B, we performed unsuccessful two-hybrid screenings and affinity chromatography on recombinant cyclin B and cyclin Bsv. This last experiment led to identification of cybip, a low-molecular mass cyclin B-binding protein.

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Experimental procedures

Handling of animals. Starfishes, *Marthasterias glacialis*, were collected by diving in the Bay of Banyuls-sur-mer (France). Preparation of prophase blocked oocytes and induction of meiotic divisions by 1-methyladenine were as previously described [4].

Recombinant proteins. Cyclin B and cyclin Bsv were produced in an insoluble form in Bl21, purified from inclusion bodies by gel filtration in 6 M GuCl, and coupled to Affigel 10 (Biorad). Cybip B and C were expressed as GST-fusion proteins. They were obtained in soluble form by cultivation of the bacteria at 25 °C and purification of the cell extracts on glutathione–Sepharose according to the manufacturer's instructions (Pharmacia).

Affinity chromatography and sequencing. M-phase extracts were prepared from *M. glacialis* oocytes treated for 40 min with 1-methyladenine. Oocytes were dissolved in 20 volumes of homogenization buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 25 mM sodium fluoride, 5 mM sodium pyrophosphate, 5 mM phenylphosphate, 0.5 mM sodium vanadate, 2 mM EGTA, 2 mM DTT, 0.2% Nonidet P 40, 1 mM benzamidine, 0.1 mg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), frozen in liquid nitrogen, and stored at −80 °C. The homogenates were sonicated centrifuged at 100,000*g* for 30 min. The extract was mixed with the affinity matrix (20 volumes to 1 volume, respectively), incubated for 2 h at 4 °C, washed and eluted, by normal homogenization buffer, then with NaCl concentration increased to 0.5 M, then 1 M plus 10% glycerol, and finally with 6 M urea and 20 mM Tris, pH 7.4.

Protein sequencing was performed at the CRBM laboratory in Montpellier (France), with a Procise sequencer (Applied Biosystem) on the proteins from urea elution, after preparative SDS–PAGE, proteolysis by trypsin, and HPLC fractionation.

cDNA cloning. Degenerated oligonucleotides based on the peptidic sequences obtained by microsequencing were used for RT-PCR amplification of a cybip DNA fragment (primers: gaYaaRtaYgaRgc NaaYga and gtNgtNgtRtaRcaYtcNcc). The full-length cDNA was obtained by RACE-PCR extension of this fragment (Smart RACE, Clonetch), with primer: gagtattggctcgtcgctttgttccctg for 3′ RACE and primer: catgccccagcgtccgtcatagtagttg for 5′ RACE.

Isoform RT-PCR analysis. Total RNA was prepared from oocytes of different starfishes using the Rneasy midi kit (Qiagen). RT-PCR was performed on each batch with the one step Titan kit (Boehringer), using a first primer common to all cybip isoforms (aacagcagtaagcc ttttgctgagcattagg), and a second primer specific for one of the three isoforms (cybip A: acgtctgcatttattttcaccgaccc, cybip B: ttgattggcatgatt ggaaaaacca, and cybip C: gtttttgcccatccctcctccagca).

Genomic DNA analysis. Starfish sperm (50 mg) was homogenized on ice in 950 ml extraction buffer (0.8 M GuCl, 1 mM Tris–HCl, pH 8, 1 mM NaCl, 1 mM EDTA, and 0.1 mM NaOH) then incubated with 40 mg/ml proteinase K overnight at 63 °C. After centrifugation at 12,000*g* for 15 min, the supernatant was added to 5 volumes of ethanol and the precipitated DNA was recovered with a glass rod and dissolved in sterile water. Each genomic DNA sample was tested for the presence of the three cybip isoforms with the same method and primers as in the RT-PCR experiments.

Immunological procedures. Polyclonal antibodies were raised in rabbits against synthetic peptides (Neosystem, Strasbourg, France) corresponding to different parts of cybip (with an additional terminal cystein for coupling purpose): MIHKLVLILSC (signal peptide), IGMIGKPAKC (cybip B specific), LPIPPPADPIDC (cybip C specific), and CRWGMYNVKGRDARV (invariant sequence), and affinity purified on the same peptides. Antibodies against *M. glacialis* cyclin B (CTSSTVMDLADQMC), cyclin Bsv (CLVASYPQLKKKIV), and the PSTAIRE region of CDKs (CEGVPSTAIREISLLKE) were produced and affinity purified in the same way. Other polyclonal antibodies were produced in rabbits and affinity purified against recombinant proteins produced in *Escherichia coli*: sea urchin (*Sphaerechinus granularis*) CDK5, *Schizosaccharomyces pombe* p13suc1.

Binding experiments. Purified cybip-GST or p13suc1 was diluted (50 μg in 1 ml) in IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM phenylphosphate, 1 mM sodium vanadate, 0.1% Triton X-100, 1 mM benzamidine, and 0.1 mg/ml SBTI) and incubated for 2 h at 4 °C with 20 μl of affinity beads (cyclin B or cyclin Bsv or BSA coupled to Affigel). After washing with IP buffer, the beads were fixed with SDS–PAGE sample buffer, for Western-blot analysis.

In pull-down experiments, glutathione–Sepharose beads were loaded with purified cybip-GST (10 mg/ml bead) in binding buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM benzamidine, and 10 μg/ml leupeptin) during 1 h at 4 °C. After washing with the same buffer, 20 μl aliquots of beads were incubated with 1 ml starfish extracts for 2 h at 4 °C, then washed with homogenization buffer, and fixed with SDS–PAGE sample buffer.

Immunofluorescence and microinjections. The jelly coat and vitelline or fertilization membrane were removed by a 20 min pronase treatment (0.5 mg/ml in seawater), followed by three rinses in bovine serum albumin (0.1% in seawater). Oocytes were extracted for 1 h in extraction buffer (20% glycerol, 50 mM MES, pH 6.7, 10 mM EGTA, 0.5 mM $MgCl_2$, and 0.5% NP40) then fixed in cold methanol/glycerol (75/25%) for at least 1 h. Anti-α tubulin (monoclonal, Amersham, dil. 1/100) or anti-cybip C (40 μg/ml) was applied overnight at 4 °C and secondary antibody for 2 h at room temperature. Observations were made with an Olympus confocal microscope.

Microinjections of purified recombinant proteins or antibody (10 mg/ml) were performed essentially according to Hiramoto [7].

Accession numbers. Cyclin B splice variant mRNA, AJ512968; cybip A, AJ512965; cybip B, AJ512966; cybip C, AJ512967.

Results

A 15 kDa protein was purified by affinity chromatography on cyclin B: its gene is polymorphic

We cloned from *M. glacialis* cDNA a cyclin B splice variant lacking highly conserved residues required for binding to cdc2 (paper in preparation). The recombinant protein was used, in parallel with wild-type cyclin B, to identify ligands of monomeric cyclin B by affinity chromatography. A protein with a relative molecular mass of 15–16 kDa, was specifically retained. Peptide microsequencing yielded 4 amino acid sequences which allowed RT-PCR and then RACE-PCR cloning of the corresponding cDNA. According to individuals, 3 different amplification products belonging to the same family were obtained, with short sequence variations but a significant identity at the amino acid level (Fig. 1). While this work was in progress, two of these three sequences were reported as *M. glacialis* p15-CDK-BP A and B [8], with p15-CDK-BP B corresponding to a protein previously purified from starfish oocytes and described as a CDK-binding protein [9]. The authors described the two isoforms as encoded by two related genes and suggested that the properties of the encoded proteins were different. We designed a series of experiments to discriminate between a multigenic family or a single gene with allelic variations.

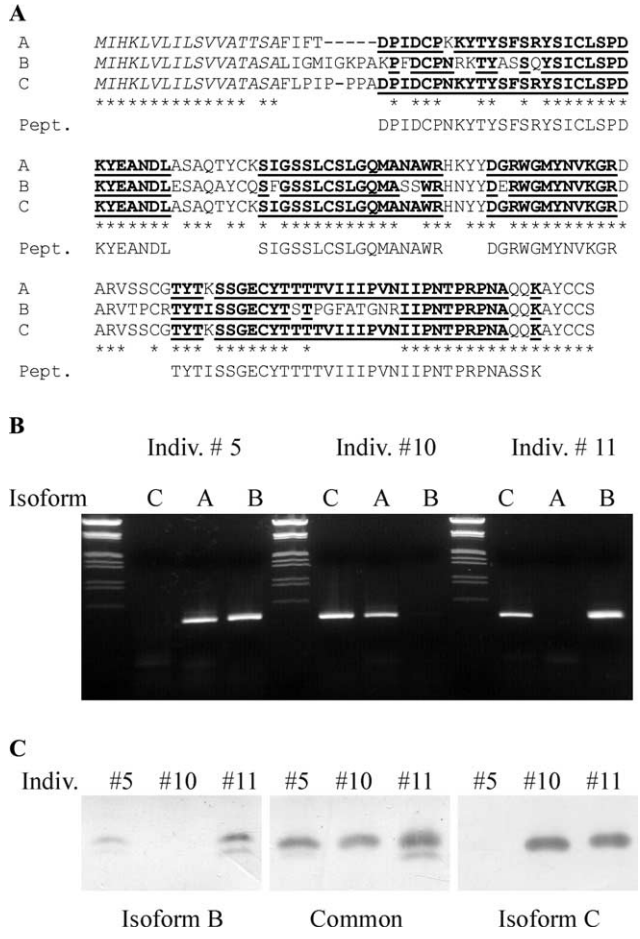

Fig. 1. Amino acid sequences of the 15 kDa protein isoforms and distribution among individual starfishes. (A) Alignment of amino acid sequences of the three isoforms (A–C) and the peptides obtained by microsequencing (Pept.). Amino acids belonging to the signal peptide sequence are shown in italic. Those similar to the microsequencing data are shown as bold underlined characters. Asterisks indicate conserved similar amino acids. The length of the fourth peptide made uncertain the identification of its last amino acids which probably explain the mismatch in the final part (QQ vs. SS). (B) Electrophoretic analysis of RT-PCR amplification of RNA extracted from oocytes of 3 *M. glacialis* individuals (#5, #10, and #11), with primers specific for the 3 isoforms (A–C). The 1% agarose gel was stained with ethidium bromide. Molecular weight markers were run on the 1st, 5th, and 9th lanes. (C) Western-blot analysis of oocyte samples for the same individuals as in (B) with antibodies specific for isoforms B, C or a common sequence. The part of the blots corresponding to proteins between 10 and 20 kDa is shown.

First, we designed specific primers to amplify each isoform separately, and raised antibodies against peptides specific for two of the proteins, and one antibody against a peptide sequence common to all three proteins (see Experimental procedures). Then, we compared the results of RT-PCR and Western blots of oocytes from several females. Individuals bearing all three isoforms were never found and almost all combinations of two isoforms were found (Fig. 1B, Table 1). Finally, antibodies recognized each time only the isoforms, and all the isoforms, that had been predicted by RT-PCR experiments (see Fig. 1C for examples). Taken together, these experiments suggest that p15 does not belong to a multigenic family, but is encoded by one single gene with at least three allelic forms.

We tried to amplify a partial sequence from individual genomic DNAs, with primer pairs specific for each isoform. As for RT-PCR experiments, amplification of the three isoforms from one individual was never obtained (Table 1), and two males out of 14 displayed only one isoform, confirming that p15 does not belong to a multigenic family but is encoded by a polymorphic gene. Alignment of PCR products shows that the chosen amplified sequence contained one intron, very similar from one allele to another (not shown).

The 15 kDa protein is a cyclin-binding protein

Vogel et al. identified p15 as a cks/suc1 distant homolog, which retained several heterologous cdks by affinity chromatography (CDK4, 5, 8, amongst others). We verified that Cdc2 (or at least a PSTAIR-containing CDK) as well as endogenous cdk5 were retained on cyclin B and cyclin Bsv columns (Fig. 2A). But the possibility that p15 was also retained as a consequence of binding to these CDKs was unlikely, since the relative abundance of p15 was far greater than that of CDKs on the column. Moreover, we found that bacterially produced recombinant p15-GST was significantly and specifically retained on bacterially produced cyclin B and cyclin Bsv (Fig. 2B), thus in complete absence of any CDK: this shows that p15 is a cyclin-binding protein. As a control in this experiment, we loaded on the cyclin B columns a solution containing bacterially produced $p13^{suc1}$, a peptide well known as a Cdc2/cdk2-binding protein [10–12], [1]: as shown in Fig. 2B, p13 was not retained on cyclin B. For this reason, and to avoid confusion with p15-cki (Ink4) or with cks, we propose to rename p15CDK-BP cybip (for cyclin-binding protein). Thus, cybip A and B correspond to p15CDK-BP A and B, respectively, and cybip C to the new allelic form we microsequenced in the first place.

Table 1
Repartition of the three allelic forms in oocyte mRNA among 13 females and in genomic DNA among 14 males

Alleles	AA	AB	AC	BB	BC	CC
In oocyte mRNA (number of females)	0	6	2	1	3	1
In genomic DNA (number of males)	0	4	2	2	6	0

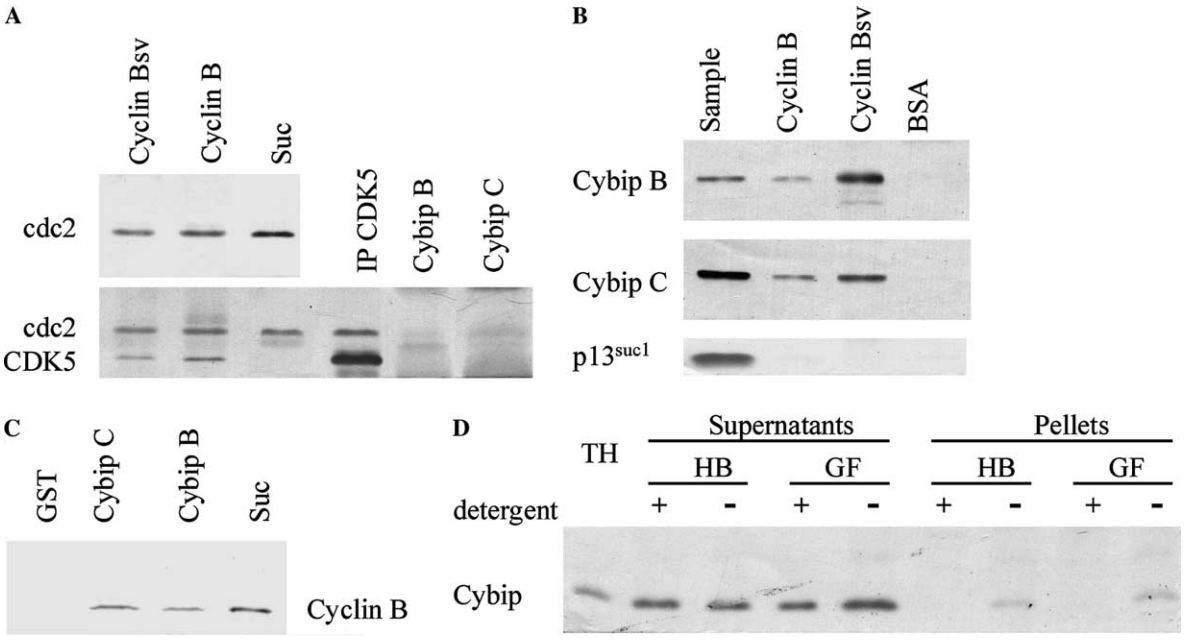

Fig. 2. Binding properties of cyclins and cybip. (A) An M-phase extract of *M. glacialis* oocytes was incubated with recombinant proteins immobilized on Affigel (cyclin Bsv, cyclin B, and $p13^{suc1}$) or glutathione–Sepharose (GST-cybip B and C), or immunoprecipitated with anti-CDK5 antibody (IP CDK5). After washing, the beads were submitted to SDS–PAGE and Western-blot analysis by anti-PSTAIR (upper panel) or anti-CDK5 (lower panel). The migration positions of cdc2 and CDK5 from the extract are indicated (since the anti-CDK5 antibody was raised against the whole protein, it also cross-reacts with cdc2). (B) Recombinant cybip B, C, and $p13^{Suc1}$ were incubated with immobilized cylin B, cyclin Bsv, and BSA. An aliquot of the sample, taken before the beginning of incubation (sample), and the washed beads were submitted to SDS–PAGE and Western-blot analysis by anti-Cybip (2 upper panels) or anti-$p13^{Suc1}$ (lower panel). (C) An M-phase extract of *M. glacialis* oocytes was incubated with recombinant proteins immobilized on glutathione–Sepharose (GST, GST-cybip B and C) or Affigel ($p13^{suc1}$). After washing, the beads were submitted to SDS–PAGE and Western-blot analysis by anti-cyclin B. For precise electrophoretic mobility comparison, the $p13^{Suc1}$ sample was diluted 10-fold. (D) Western blot from ultracentrifugation supernatants and pellets of oocyte extracts in two buffers, with or without detergent. Aliquots of oocytes from a single starfish were dissolved in SDS sample buffer (TH), or the homogenization buffer used for affinity chromatography (HB) with (+) or without (−) NP40, or the gel filtration buffer (GF) with (+) or without (−) CHAPS. After ultracentrifugation for 30 min at 200,000*g*, aliquots of each supernatant and each pellet were dissolved in SDS sample buffer and used for Western-blot analysis with anti-cybip antibody. The portion of the blot corresponding to proteins of molecular weight between 14 and 20 kDa is shown.

Fig. 2B also shows that the two bacterially produced cybip alleles bind equally to the recombinant cyclins, suggesting that their affinities for cyclin B are equivalent. This observation was strengthened by the fact that both isoforms were recovered from homogenates of different females (not shown).

As the three antibodies we had raised were found inefficient for immunoprecipitation, we performed cybip-GST pull-down to confirm the association of oocyte extract cyclin B and GST-tagged cybip. Fig. 2C shows that cyclin B was equally detected on cybip B and C bound to glutathione beads.

The N-terminus of cybip ORF encodes a signal peptide, which is cleaved in the mature protein

Signal-peptide prediction (SignalIP V2.0, [13,14]) identified a signal peptide with a 99.7% probability of cleavage in the mature protein. A peptide corresponding to this N-terminal sequence was used to produce and affinity purify a specific antibody which did not recognize the native protein in whole homogenates, nor the material retained by affinity on cyclin B (not shown), confirming that the signal peptide is cleaved whatever the expressed allele.

Some programs predicted that cybip should be exported after addressing to the membrane and cleavage. We analyzed by Western blotting the 100,000 g supernatant and pellet fractions of homogenates made in two different extraction buffers and in presence or absence of detergents. Fig. 2D shows that most of the cybip protein was found in the 100,000 g supernatant, even in the complete absence of detergent. Moreover, in gel filtration on a Superose 6 FPLC column, cybip was eluted in the low-molecular mass fractions (not shown). This shows that the majority of cybip protein is cytosolic and probably not associated with cyclin B.

Cybip accumulates on the metaphase spindle and cleavage furrow

For immunofluorescence, we first checked by Western blots oocytes from several starfishes for the presence of the various allelic forms. The results presented here were obtained from a female expressing isoform C. Results did not vary according to the isoforms present in the oocytes

(not shown). Immunofluorescence of maturing oocytes showed a concentration of the protein on the mitotic apparatus and at the animal pole cortex surrounding the outer pole of the spindle. At the time of first polar body emission, cybip was observed concentrated as a ring corresponding to the cleavage furrow (Figs. 3A and B) and this localization persisted after cytokinesis. The same figure was observed with a primary antibody specific for the isoform C and with an antibody recognizing all three isoforms (Figs. 3C and D).

Cybip is involved in H1 kinase deactivation at the end of M phase

In order to avoid competition between isoforms, we worked on starfishes producing cybip isoform C

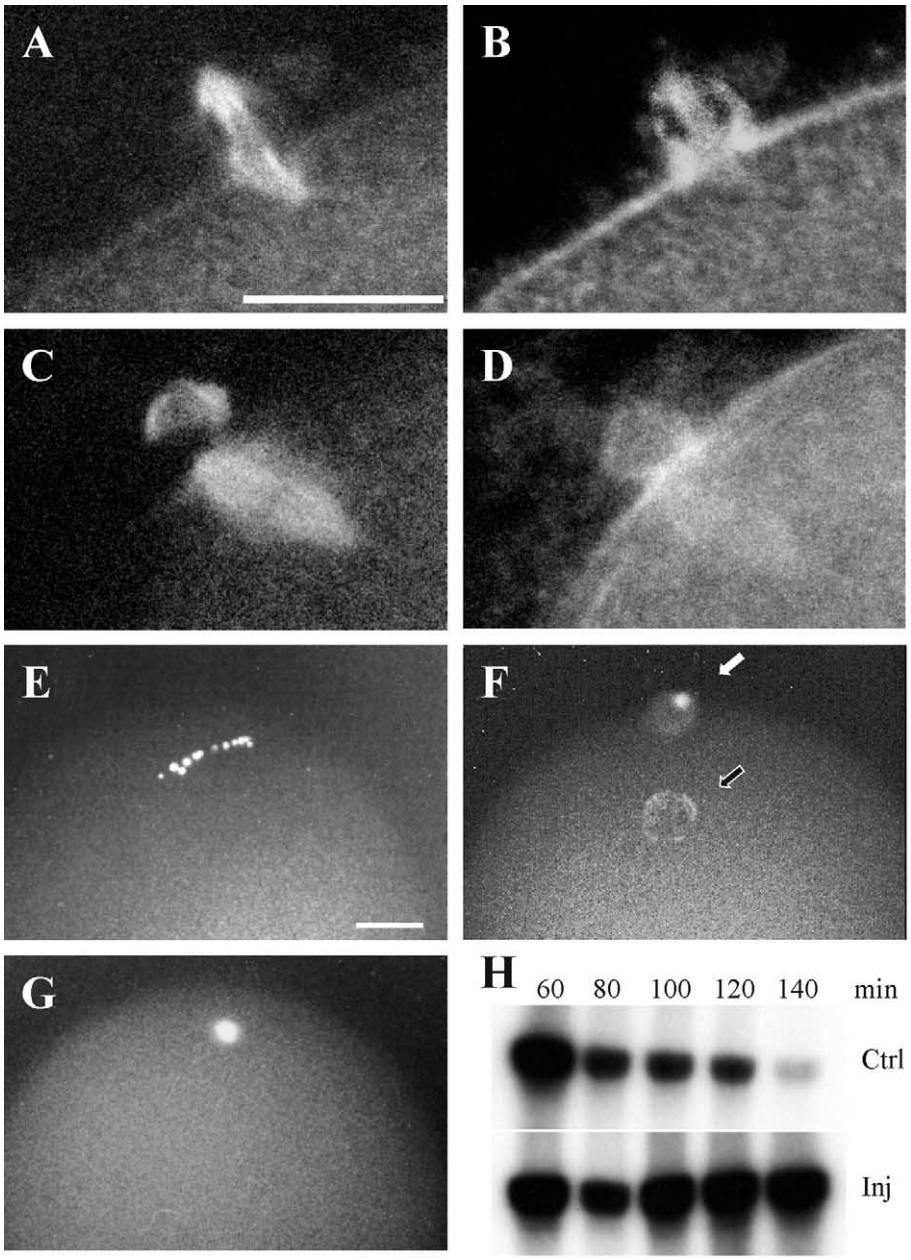

Fig. 3. Immunolocalization of cybip and effect of cybip or anti-cybip microinjection on oocyte maturation. *M. glacialis* oocytes were treated with detergent and fixed at 90 min post-hormone addition (first polar body emission), followed by double staining with FITC for anti-tubulin (A and C) and Texas red for anti-cybip C (B) or anti-cybip common sequence (D). Scale bar = 20 μm. Hoechst DNA-staining of *M. glacialis* mature oocytes, microinjected with GST-cybip C (E), GST (F) or anti-cybip C antibody (G). Oocytes were microinjected at the germinal vesicle stage, then induced to mature, and fixed after 180 min. White arrow shows the two polar bodies (out of focus) and the black arrow the reformed pronucleus in a control GST-microinjected oocyte. Scale bar = 20 μm. (H) Autoradiography of histone H1 phosphorylation in the presence of [γ-^{32}P]ATP, by extracts of GST (Ctrl) or GST-cybip C microinjected (Inj.) oocytes fixed at various times after hormone addition (min). Control oocytes emitted first polar body at 80 min and second polar body at 110 min.

(cybip C) and with the corresponding antibody and recombinant GST-chimeric protein.

GST-cybip C was purified on gluthatione–Sepharose then concentrated at 10 mg/ml (230 μM). The final concentration of the protein in the cell after microinjection was approximately 6.4 μM, thus probably far over the physiological concentration. Control microinjections of similar GST concentrations were without effect on the progression of cell cycle. Cybip C microinjected oocytes underwent maturation when triggered by 1-methyladenine, the natural hormone inducing maturation in starfish. Cybip apparently did not change the dose nor the timing of response to the hormone (not shown) and thus did not interfere with MPF activation. First polar body was never emitted, even after 5 h when oocytes began to die. DNA staining performed at 3 h post-hormone addition showed that chromosomes remained condensed and rather dispersed at animal pole (Fig. 3E). In contrast in controls both polar bodies had been emitted and the pronucleus was reformed (Fig. 3F). Similar results were obtained after microinjection of the antibody specific for the C isoform, except that chromosomes were reproducibly grouped at animal pole (Fig. 3G). H1 kinase activity was also maintained high in GST-cybip C-microinjected maturing oocytes (Fig. 3H), as well as in oocytes that were microinjected with anti-cybip C antibody (not shown), whereas controls showed the usual reduction and final disappearance of activity at first and 2nd polar body emission. We also checked, by cytoplasmic transfers, that GST-cybip C microinjected oocytes retained a high MPF level 3 h after hormonal stimulation, at difference to GST micr-oinjected controls (not shown). Western-blot analysis of cybip during meiosis completion and early development, up to the blastula stage, showed no difference in its abundance or electrophoretic mobility (not shown).

Discussion

In this paper, we show that proteins implicated in cell cycle progression can bind cyclin B, even in the absence of cdks: a similar observation has already been made concerning cdc25, the H1-kinase activating phosphatase [15], in an experiment which has not been confirmed later [16]. As shown on Fig. 2, a PSTAIR-containing cdk, probably Cdc2, and also CDK5 are retained both on cyclin B and cyclin Bsv, from starfish oocyte cytosol. While the amount of these cdks is far lower than the amount of cybip retained on the same column, we cannot rule out the possibility that they can bind directly cybip, as was previously suggested [8,9]. But the present demonstration that bacterially produced GST-cybip binds specifically recombinant cyclin B and cyclin Bsv definitively shows that cybip is at least a cyclin B-binding protein, and that the cybip-binding sequence in cyclin B is not the C-terminal alpha helix, lacking in the variant. Actually, cyclin Bsv seems to have more affinity for cybips than normal cyclin B (Fig. 2B).

We also show that cybip is encoded by a polymorphic gene, but we did not find any functional difference among allelic isoforms, at least between isoforms B and C, for which we had obtained both antibodies and recombinant proteins. Moreover, the fact that any combination of isoforms can be found in genomic DNA from a starfish population, and that one or two isoforms can be absent in a given individual, suggests that, if the function of cybip is essential, the essential properties of all isoforms are identical.

An intriguing observation is that the N-terminus of all isoforms is predicted to be a signal peptide and appears to be effectively cleaved in the cell; moreover, while the protein is predicted with a high probability to be externalized, we found it in the cytosolic fraction, absolutely devoid of membranes.

We did not reach our first aim of identifying activators of pre-MPF able to bind monomeric cyclin B: clearly, cybip is not involved in the mechanisms which lead to dephosphorylation of the T14 and Y15 of Cdc2. Indeed, our current observations, both in immunofluorescence and in microinjection experiments, suggest an involvement of cybip in exit from M phase. While the detergent-resistant fraction of the protein is localized both on the spindle and in the animal cortex during metaphase, it is found mainly on the cleavage furrow at telophase-cytokinesis: this suggests that part of the microtubule-bound fraction was destroyed or changed localization upon M phase exit. The localization of cybip on the spindle and at spindle poles, and then on furrow is in agreement with a regulatory or structural role in M-phase exit and polar body formation. Indeed, we show that microinjection of either an excess of recombinant protein or the antibody impedes both polar body formation and cyclin B-dependent H1-kinase inactivation. This common effect of the protein in excess and the antibody directed against the same protein strongly suggests that the function of cybip with regard to cyclin B is regulated by binding to a third partner protein "X." The formation of the trimer cyclin B–cybip-"X" would lead to cyclin B destruction, either because cybip is an inhibitor of cyclin B degradation (the dimer cyclin B–cybip cannot be degraded), and "X" inhibits cybip; or because cybip is an activator of cyclin B degradation, whose function requires binding to "X," which could be a component of the poly-ubiquitination pathway, or of the 26S-proteasome, for example.

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