Fizzy is required for activation of the APC/cyclosome in Xenopus egg extracts

Thierry Lorca, Anna Castro, Anne-Marie Martinez, Suzanne Vigneron, Nathalie Morin, Stephan Sigrist1, Christian Lehner1, Marcel Doreé2 and Jean-Claude Labbé

Centre de Recherches de Biochimie Macromoléculaire, CNRS UPR 1086, 1919 route de Mende, 34293 Montpellier Cedex 5, France and 1Department of Genetics, University of Bayreuth, 95440 Bayreuth, Germany
2Corresponding author e-mail doree@crbm.cnrs-mop.fr

The Xenopus homologue of Drosophila Fizzy and budding yeast CDC20 has been characterized. The encoded protein (X-FZY) is a component of a high molecular weight complex distinct from the APC/cyclosome. Antibodies directed against FZY were produced and shown to prevent calmodulin-dependent protein kinase II (CaMKII) from inducing the metaphase to anaphase transition of spindles assembled in vitro in Xenopus egg extracts, and this was associated with suppression of the degradation of mitotic cyclins. The same antibodies suppressed M phase-promoting factor (MPF)-dependent activation of the APC/cyclosome in interphase egg extracts, although they did not appear to alter the pattern or extent of MPF-dependent phosphorylation of APC/cyclosome subunits. As these phosphorylations are thought to be essential for APC/cyclosome activation in eggs and early embryos, we conclude that at least two events are required for MPF to activate the APC/cyclosome, allowing both chromatid segregation and full degradation of mitotic cyclins. The first one, which does not require FZY function, is the phosphorylation of APC/cyclosome subunits. The second one, that requires FZY function (even in the absence of MAD2 protein and when the spindle assembly checkpoint is not activated) is not yet understood at its molecular level.

Keywords: APC/cyclosome/Drosophila/Fizzy/Xenopus

Introduction

Progression of the eukaryotic cell cycle is controlled by the activity of the CDK/cyclin kinases (Murray and Hunt, 1993). For example, M phase-promoting factor (MPF), which drives the cell cycle into M phase, is activated at G2/M by dephosphorylation of inhibitory residues on cdc2 (CDK1) and inactivated at the end of M phase by proteolysis of its cyclin B subunit (Nurse, 1990; Glotzer et al., 1991). The degradation at the metaphase–anaphase transition of PDS1/CUT2 (Cohen Fix et al., 1996; Funabiki et al., 1996; Yamamoto et al., 1996a,b) and related proteins, which allows sister chromatid segregation, as well as that of mitotic cyclins A and B, depends on a complex pathway that targets these substrates, through the attachment of polyubiquitin chains, for destruction by the 26S proteasome (reviewed in Hochstrasser, 1995; King et al., 1996). In this pathway, ubiquitin is first activated by formation of a thioester bond with the ubiquitin-activating enzyme E1. Ubiquitin is then transsterified to one member of a family of ubiquitin-conjugating (UBC) enzymes. In a third step, ubiquitin is transferred from the UBC enzyme to lysine residues of the target protein by a ubiquitin–protein ligase, the anaphase-promoting complex (APC) or cyclosome (King et al., 1995; Sudakin et al., 1995). The APC/cyclosome is a 20S/36S particle, many components of which have now been identified by biochemical and genetic studies in several organisms (Peters et al., 1996; Zachariae et al., 1996, 1998; Yu et al., 1998).

This degradation pathway is inactive during S, G2 and early M phase, and this is required for the accumulation of mitotic cyclins and cohesion of sister chromatids. Then its activity increases, allowing sister chromatid segregation and exit from M phase. However, the molecular mechanisms responsible for this periodic activation are poorly understood. As neither the activities of E1, E2 nor that of the proteasome have been shown to change during the cell cycle, in contrast to that of the APC/cyclosome, this particle is believed to be the target of cell cycle-dependent regulation. Cyclin B–cdc2 kinase has been shown to turn on the cyclin degradation pathway in extracts prepared from Xenopus eggs (Felix et al., 1990), and this correlates with activation of the APC/cyclosome and extensive phosphorylation of, at least, CDC27 and CDC16 (CUT9 in Schizosaccharomyces pombe), two subunits of the APC which have tetratricopeptide repeats (Goebel and Yanagida, 1991; King et al., 1995; Yamada et al., 1997). As dephosphorylation inactivates the purified APC/cyclosome (Sudakin et al., 1995; Peters et al., 1996), this strongly suggests that, at least in the early development of higher eukaryotes, the APC/cyclosome is activated by mitotic phosphorylation of CDC27/CDC16 and possibly other subunits. In contrast, there is, as yet, no available evidence in budding yeast for cell cycle-dependent phosphorylation of any APC subunit, even though APC activity is also cell cycle regulated.

Apart from phosphorylation, activation of the ubiquitin–proteasome pathway appears to involve members of a family of proteins containing seven WD-40 repeats in their C-terminus, in both yeast and higher eukaryotes. In Drosophila, these proteins are Fizzy (FZY) and Fizzy-related (FZR). FZY is required for progression beyond metaphase and mitotic cyclin degradation (Dawson et al., 1993, 1995; Sigrist et al., 1995), while FZR is required for cyclin degradation during G1 when the embryonic epidermal cell proliferation stops, and during G2 preceding...
Fig. 1. Comparison of the amino acid sequences derived from cDNAs of Xenopus Fizzy (X-FZY), Drosophila Fizzy (D-FZY) and Drosophila Fizzy-related (D-FZR). Regions with identity between the X-FZY and at least one of the two other sequences are in bold. The seven WD-40 repeats in the C-terminal region are indicated by numbered arrows. The comparison was generated with the MAP program (Xiaoqiu, 1994).

Results

Identification of a Xenopus Fizzy (X-fzy1) homologue

We have isolated a cDNA encoding a Xenopus homologue of Fizzy/CDC20. This X-fzy1 cDNA (DDBJ/EMBL/GenBank accession No. AF 034578) contains an open reading frame (ORF) coding for a protein with 507 amino acids and a predicted molecular weight of 55 kDa. The amino acid sequence shares 52 and 46% identity with Drosophila Fizzy and budding yeast Cdc20p, respectively. X-FZY1 is more similar to Drosophila Fizzy (D-FZY) than to Drosophila Fizzy-related (D-FZR) (Figure 1). Conversely, the recently identified fizzy-related Xenopus gene product (X-FZR) is more similar to D-FZR than to D-FZY (Sigrist and Lehner, 1997). All these proteins are similar in their C-terminal domain, which contains seven WD-40 repeats.

Production and characterization of polyclonal antibodies that inactivate X-FZY

Polyclonal antibodies, raised in rabbit against a fusion protein of GST with truncated X-FZY (residues 70–507: GST–FZYΔ69N), were affinity purified on a fusion protein of the Escherichia coli maltose-binding protein with the same X-FZY fragment. Affinity-purified antibodies against X-FZY detected a protein in extracts prepared from unfertilized frog eggs (Figure 2A, left panel, lane 1) which had the same apparent molecular weight as in vitro translated X-FZY (lane 2). The anti X-FZY antibodies did not recognize the in vitro translated X-FZR (lane 3). Conversely, affinity-purified antibodies raised against an N-terminal peptide of X-FZR (residues 2–18) readily recognized (Figure 2A, right panel) in vitro translated X-FZR (lane 3), but failed to recognize either in vitro translated X-FZY (lane 2) or any protein in frog egg extracts (lane 1). As FZR antibodies recognize FZR as efficiently as FZY antibodies recognize FZY (see legend to Figure 2A), it appears that the FZR protein is not present at a detectable level in frog eggs. In fact, we failed to detect the FZR protein before the mid-blastula transition (not shown), when a G1 phase first appears in the cell cycle after a series of embryonic cell cycles consisting exclusively of alternating S and M phases (Signoret and Lefresne, 1971; Gerhart, 1980; Newport and Kirschner, 1982). By Western blotting, in comparison with the recombinant GST–FZYΔ69N, the concentration of X-FZY was estimated to range from 40 to 100 nM in unfertilized frog eggs.
interphase egg extract (Figure 2B), to migrate then with the same mobility as observed in an extract prepared from unfertilized eggs, whereas X-FZR is not detected. Lane 1, extract prepared from a CSF extract (75 μg of proteins); lane 2, reticulocyte lysate (2 μl) programmed with X-FZY (estimated amount of [35S]-labelled X-FZY: 0.4 pmol); lane 3, reticulocyte lysate (2 μl) programmed with X-FZR (estimated amount of [35S]-labelled X-FZR: 0.2 pmol). The left blot was probed with affinity-purified antibodies directed against X-FZY, the right blot with affinity-purified antibodies directed against X-FZR. (B) Analysis by Western blotting of the changes in the electrophoretic mobility of X-FZY during the cell cycle. 1, interphase extract (prepared 40 min after parthenogenetic activation); M, the same interphase extract after incubation for 15 min with highly purified starfish cyclin B–cdc2 kinase (1 μl into 20 μl; volume-specific activity: 200 pmol P transferred to H1 histone/min/μl); CSF, CSF extract; CSF + CaMKII, the same CSF extract after 45 min incubation with in vitro translated, constitutively active CaMKII (2 μl reticulocyte lysate into 20 μl CSF extracts). (C) [γ-32P]ATP (50 μCi) was added simultaneously with the kinase. X-FZY and co-immunoprecipitated proteins were analysed by autoradiography after immunoprecipitation with affinity-purified X-FZY antibodies.

When a CSF extract was fractionated by gel filtration on a Superdex 200 column, phosphorylated FZY was recovered in a peak of high molecular weight even in the presence of 1% Triton and 0.5% deoxycholate (Figure 3A, lower panel), indicating its stable association with other components in a macromolecular complex. Even though not separated from the APC/cyclosome by gel filtration only (Figure 3A, upper panel), this FZY-containing complex is obviously distinct from the APC/cyclosome, as the material immunoprecipitated by FZY antibodies does not contain CDC27, and conversely the APC/cyclosome complex immunoprecipitated by CDC27 antibodies does not contain FZY (Figure 3B). In this experiment, as in the following ones (see below, Figure 6C), it was checked that CDC27 immunoprecipitates are functional and support cyclin B ubiquitination.

FZY is required to exit from CSF arrest

The WD-40 repeats have been shown to provide protein–protein interaction faces, at least in trimeric G protein complexes (Wall et al., 1995; Lambright et al., 1996). As the polyclonal FZY antibodies were obtained from animals immunized against a truncated protein comprising seven of these domains, they might be expected to impair FZY function. We investigated their effect on the metaphase–anaphase transition in Xenopus egg extracts, using in vitro assembled spindles (Holloway et al., 1993). Sperm nuclei were first allowed to replicate DNA in an interphase egg extract, then a CSF extract was added to drive and arrest them at metaphase. When constitutively active CaMKII was added, anaphase was triggered, as previously described (Morin et al., 1994). The same result was obtained when control γ-globulins (or a variety of antibodies unrelated to FZY) were added prior to CaMKII. At 45 min after CaMKII addition, spindles had disassembled in control extracts (Figure 4) and a network of long interphase, centrosome-nucleated microtubules (upper middle micrograph), and karyomeres (lower left micrograph) were observed, which subsequently fused to reconstitute an interphase nucleus. In contrast, chromatids failed to segregate and spindles remained arrested at metaphase when affinity-purified FZY antibodies were added before CaMKII (Figure 4, upper and lower right micrographs). We never observed interphase-like microtubules in extracts containing FZY antibodies.

The above result suggested that antibodies had prevented FZY from performing an essential function for triggering chromatid segregation and rearrangement of spindle microtubules after metaphase. This function could be the activation of the ubiquitin-dependent proteolytic pathway responsible for degradation of both mitotic cyclins and the unidentified Xenopus homologue of PDS1/CUT2, which is likely to control sister chromatid cohesion.

To investigate this possibility, constitutively active CaMKII was assayed for its ability to trigger degradation of [35S]-labelled cyclin B1 in a CSF extract to which affinity-purified FZY antibodies, or control immunoglobulins (Mock Ab), had been added. As shown in Figure 5A, CaMKII failed to induce degradation of mitotic cyclins in the first, but not in the second case. The effect of FZY antibodies was specific, as their ability to block cyclin degradation was lost after several freeze–thaw cycles or after pre-incubation with the antigen used for immunization (not shown). Moreover, the block of cyclin degradation was also obtained (Figure 5B) when FZY was quantitatively depleted from egg extracts (Figure 5C) prior to the addition of CaMKII using either the polyclonal antibody directed against the N-terminally truncated FZY fusion protein or a polyclonal antibody raised against the 15 amino acid N-terminal peptide of X-FZY. Another way to overcome the block to cyclin degradation in CSF extracts is to treat them with 1 μM okadaic acid or microcystin (Lorca et al., 1991). As shown in Figure 5D, the phosphatase inhibitors also failed to activate cyclin...
Fig. 3. X-FZY is a component of a high molecular weight complex distinct from the APC/cyclosome. (A) Side-by-side analysis by Western blot of consecutive fractions (30 μl loaded), after chromatography of a CSF extract by gel filtration on a Superdex 200 column in the presence of 1% Triton X-100 and 0.5% Na deoxycholate. Upper panel: blot probed with affinity-purified antibodies directed against CDC27. Lower panel: blot probed with affinity-purified antibodies directed against FZY. The vertical arrows indicate the positions of elution for thyroglobulin (670 kDa), catalase (230 kDa) and ovalbumin (43 kDa). (B) Fractions corresponding to the peak of high molecular weight FZY complex were pooled and 400 μl immunoprecipitated without dilution with affinity-purified CDC27 (IP CD27) or FZY (IP FZY) antibodies cross-linked to protein A–Sepharose. Materials eluted from each matrix were analysed by Western blotting with a mixture of affinity-purified CDC27 and FZY antibodies.

Fig. 4. FZY antibodies prevent in vitro assembled spindles from exit of metaphase arrest in response to CaMKII. A suspension (20 μl) of in vitro assembled spindles, arrested at metaphase by CSF, was treated for 15 min with affinity-purified FZY antibodies (Ab Fizzy) or the same amount (2 μg) of rabbit immunoglobulins (CT: control). Constitutively active, in vitro translated CaMKII (CaMK) was added (2 μl). Samples were taken before, or 45 and 90 min after CaMKII addition, and examined by fluorescence microscopy. Chromosomes were visualized by the fluorescence of the DNA-binding dye Hoechst 33342, and microtubules with rhodamine-labelled tubulin. The bar in the lower right micrograph corresponds to 2.5 μm (same magnification for all micrographs).
were also analysed by Western blotting with affinity-purified FZY antibodies (C) to confirm depletion of the FZY protein.

Although the antibodies did purified cyclin B–cdc2 kinase, prepared from starfish interphase egg extract prior to the addition of highly affinity-purified FZY antibodies were added to an dependent activation of the cyclin degradation pathway, shown). Finally, no degradation of budding yeast PDS1 could be detected in extracts containing FZY antibodies (not shown).

**FZY is required for MPF-dependent activation of the APC/cyclosome**

In *Xenopus* egg extracts, mitotic cyclins are stable at interphase, but addition of purified cyclin B–cdc2 kinase can trigger cyclin degradation. There is a lag phase of ~15 min after kinase addition before cyclin degradation commences, and this lag phase cannot be shortened by increasing cyclin B–cdc2 kinase activity (Felix et al., 1990). This suggests that activation of the APC/cyclosome, which is believed to be rate limiting for cyclin degradation, may not depend only on cdc2 kinase-dependent phosphorylation, but may involve other events. Consistent with this view, maximal phosphorylation of the CDC27 subunit occurs earlier than activation of the APC/cyclosome in egg extracts (King et al., 1995).

To investigate the possible involvement of FZY in MPF-dependent activation of the cyclin degradation pathway, affinity-purified FZY antibodies were added to an interphase egg extract prior to the addition of highly purified cyclin B–cdc2 kinase, prepared from starfish oocytes (Labbé et al., 1991). Although the antibodies did not reduce cyclin B–cdc2 kinase activity, they completely suppressed degradation of both cyclins A and B (Figure 6A). MAP kinase has also been shown to prevent MPF from turning on the cyclin degradation pathway (Abriou et al., 1996) and proposed to mediate inhibition of the metaphase–anaphase transition by the spindle assembly checkpoint (Minshull et al., 1994; Wang et al., 1997). However, the FZY antibodies failed to activate MAP kinase (Figure 6B).

To assess FZY function more directly in the above experiments, the APC/cyclosome was immunoprecipitated from egg extracts treated with FZY or control antibodies before addition of cyclin B–cdc2 kinase. Its ability to conjugate ubiquitin to 35S-labelled cyclin B1 in the presence of E1 and UBC-X was assayed. As shown in Figure 6C, APC/cyclosome immunoprecipitated from an egg extract containing the same amount of affinity-purified FZY antibodies was inefficient in generating cyclin B1 conjugates (lane 3). In contrast, APC/cyclosome immunoprecipitated from an egg extract containing the same amount of affinity-purified FZY antibodies was inefficient in generating cyclin B1 conjugates (lane 2). In fact, it was as inefficient as APC/ cyclosome isolated from a non-MPF-treated interphase extract (lane 1). This indicates that FZY function is required for MPF to activate the APC/cyclosome. The same results were obtained when FZY was quantitatively depleted from egg extracts prior to the addition of cyclin B–cdc2 kinase (not shown).

In the above experiment, cyclin B–cdc2 kinase reduced the electrophoretic mobility of CDC27 to the same extent, suggesting identical phosphorylations, when added to control egg extract or to extract in which FZY function had been impaired with blocking antibodies (Figure 6D, 0.5 versus 15 min). This was confirmed by directly monitoring 32P incorporation into the APC/cyclosome following MPF addition (Figure 6E). As phosphorylation of the APC/cyclosome is known to be essential for its activation in egg extracts (Sudakin et al., 1995; Peters et al., 1996), we conclude that at least two events are required for MPF to activate the APC/cyclosome complex. The first one, which does not require FZY function, is the phosphorylation of CDC27, and possibly other subunits.
Fig. 6. FZY antibodies suppress MPF-dependent activation of the APC/cyclosome. (A) An interphase extract (20 μl), prepared 40 min after parthenogenetic activation from Xenopus eggs, received either 2 μg of affinity-purified FZY antibodies, or the same amount of rabbit immunoglobulins (Mock Ab). After 10 min, highly purified starfish cyclin B–cdc2 kinase was added (1 μl; volumic activity: 200 pmol P/min/μl). Samples were taken 0.5, 15 and 40 min thereafter and analysed by Western blotting with an antibody directed against starfish cyclin B (upper panel) for degradation of cyclin B. The lower panel shows the same experiment, except that recombinant human cyclin A was added before cyclin B–cdc2 kinase addition, and samples were analysed by Western blotting with an antibody directed against human cyclin A. (B) Samples taken 30 min after cyclin B–cdc2 kinase addition in the above experiment, in the presence of affinity-purified FZY antibodies (lane 2) or mock immunoglobulins (lane 3), were run together with a CSF sample (lane 1) and analysed by Western blotting for detection of the active, phosphorylated form of MAPK. Active MAPK is detected in CSF extract only. (C) Samples, taken before (lane 1) or 20 min after addition of starfish cyclin B–cdc2 kinase in the experiment depicted in (A), and containing affinity-purified FZY antibodies (lane 2) or mock immunoglobulins (lane 3), were first depleted with protein A–Sepharose, then supernatants were immunoprecipitated with affinity-purified CDC27 antibodies. The immunoprecipitated APC/cyclosome was assayed in each condition for accumulation of ubiquitin conjugates from 35S-labelled cyclin B1 during a 15 min incubation (see Materials and methods). (D) Same experiments as (A), but the blot was probed with affinity-purified CDC27 antibodies. (E) Same experiment as in (A), using an interphase extract (20 μl) containing 2 μg of FZY antibodies (+) or 2 μg of control immunoglobulins (–), but starfish cyclin B–cdc2 kinase was added simultaneously with [γ-32P]ATP (50 μCi). After 20 min, FZY antibodies or control immunoglobulins were depleted with protein A–Sepharose beads, then the APC/cyclosome was immunoprecipitated with affinity-purified CDC27 antibodies and analysed by autoradiography after SDS–PAGE.

of the APC/cyclosome. The second one, which requires FZY function, is not yet understood at its molecular level.

Although the above results showed that FZY is required for APC/cyclosome activation, they did not address the question as to whether FZY is still required later on to maintain APC/cyclosome activity. To investigate this question, GST–cyclin B was added to an interphase extract. The recombinant cyclin associates with endogenous cdc2 and activates the ubiquitin-dependent degradation pathway but, as a consequence of its indestructibility (due to the N-terminal GST moiety), cdc2 kinase is not inactivated and the degradation pathway remains permanently turned on (Morin et al., 1994; Abrieu et al., 1996). In the next experiment, an interphase extract received affinity-purified FZY antibodies (or the same amount of mock antibodies) either before GST–cyclin B, or after the indestructible GST–cyclin B had turned on the cyclin degradation pathway. In both cases, we confirmed that GST–cyclin B readily associates with cdc2 and produces similar levels of H1 kinase activities (not shown). As shown in Figure 7, FZY antibodies completely prevented GST–cyclin B from turning on the cyclin degradation (right upper panel, lanes A) if added before or simultaneously with the indestructible cyclin, and this was correlated, as expected, with poor activation of the APC/cyclosome (right lower panel, lanes A). Although FZY antibodies also reduced APC activation to some extent and slowed down cyclin degradation if added after the indestructible cyclin had
turned on the ubiquitin-dependent degradation pathway (lanes C, as compared with control lanes B), this effect was much less pronounced than observed when FZY function was blocked earlier. We conclude that FZY is required mainly for APC/cyclosome activation.

**X-MAD2 is not required for the block of cyclin degradation induced by impairing FZY function**

The Fizzy homologues CDC20 in budding yeast and Slp1 in fission yeast recently have been shown to be effectors of the MAD2-dependent spindle checkpoint (Hwang et al., 1998; Kim et al., 1998). To investigate if the only function of Fizzy is to serve as a target of MAD2 in the spindle assembly checkpoint, a CSF extract was quantitatively depleted of X-MAD2 before addition of affinity-purified FZY antibodies (Figure 8A, upper panel). Even in the absence of X-MAD2, the FZY antibodies specifically prevented microcystin from triggering degradation of mitotic cyclins (Figure 8B). Moreover, most FZY complexes seem to be free of any association with X-MAD2, as they remain in the supernatant when egg extracts are quantitatively depleted of X-MAD2 (Figure 8A, lower panel). We conclude that Fizzy is not merely a target of MAD2 in the spindle assembly checkpoint, and that it plays a more general role in activation of the APC/cyclosome. Even though MAD2 was reported to bind the APC/cyclosome when the spindle checkpoint is activated in HeLa cells (Li et al., 1997), we never detected X-MAD2 in CDC27 immunoprecipitates, even from extracts treated with FZY antibodies (Figure 8C).

**Discussion**

In this study, we have identified a new member of the Fizzy subfamily of proteins, *Xenopus* X-FZY. Unlike the previously identified Fizzy-related protein X-FZR, which is not translated before the mid-blastula transition, X-FZY is detected readily in oocytes and early embryos. In *Drosophila* also, FZR is not expressed before the thirteenth embryonic mitosis, unlike FZY (Sigrist and Lehner, 1997). Translation of only X-FZY in the early embryonic cell cycle allowed us to investigate, in the absence of overlap with X-FZR, what function(s) may be performed specifically by FZY.

Recently, extensive work in budding yeast has led to the proposal that the APC/cyclosome may distinguish between different substrates through the action of the Fizzy-related protein HTC-1/CDH1 and that of CDC20, which is most similar to Fizzy. Indeed cells deficient for HTC-1/CDH1 were impaired in their ability to degrade mitotic cyclins (Schwab et al., 1997; Visintin et al., 1997), while cells deficient for CDC20 were found to be impaired in their ability to degrade the anaphase inhibitor PDS1, but not mitotic cyclins (Visintin et al., 1997). Although this interpretation is consistent with experimental evidence in budding yeast, it is difficult to reconcile with the observation that Fizzy-related proteins appear to be absent during early development of *Drosophila* and *Xenopus*. Fizzy proteins, therefore, play a more basic role in regulation of APC/cyclosome activity in higher eukaryotes, allowing both sister chromatid separation and mitotic cyclin degradation. Fizzy-related proteins may function specifically to extend the period of APC/cyclosome activity when a G1 phase appears during development.

We have now identified FZY as a component of the cell machinery that is required, in addition to phosphorylation of CDC27 (and possibly that of other subunits of the APC/cyclosome), for MPF to convert the APC/cyclosome from its inactive, interphase state, to its active, mitotic...
state. Specifically, we have shown that, even though MPF-dependent phosphorylation of CDC27 and other subunits was not altered, the APC/cyclosome was refractory to MPF activation in the presence of FZY-blocking antibodies in cell cycle extracts induced to enter mitosis. The APC/cyclosome could not be recovered in an active form by immunoprecipitation, in contrast to the APC/cyclosome incubated with mock antibodies, which readily directed ubiquitination of mitotic cyclins. As a consequence, MPF failed to trigger degradation of cyclins A and B, which it normally does in egg extracts. In contrast to its dramatic effect in suppressing MPF-dependent activation of the APC/cyclosome, the FZY-blocking antibodies had a less pronounced inhibitory effect when added once the cyclin degradation pathway had been turned on. Thus, FZY appears to impinge on the APC/cyclosome mainly at the level of its mitotic activation. In contrast to HCT-1/CDH1, which controls degradation of mitotic cyclins only after sister chromatid segregation in budding yeast, FZY is already required for the degradation of cyclin A, which occurs before the onset of chromatid segregation in higher eukaryotes (Minshull et al., 1990; Pines and Hunter, 1991; Pagano et al., 1992).

Suppression of CDC20 and FZY function has been shown to alter microtubule dynamics in budding yeast and *Drosophila*, respectively (Sethi et al., 1991; Dawson et al., 1995; O’Toole et al., 1997). In budding yeast, overexpression of the Fizzy-related HCT-1/CDH1 allowed cells to escape from the spindle assembly checkpoint (Schwab et al., 1997; Visintin et al., 1997). Complexes between MAD and CDC20/Sil1 have also been demonstrated to be essential for activation of the spindle assembly checkpoint in budding and fission yeast (Hwang et al., 1998; Kim et al., 1998). These results raise the possibility that suppression of APC/cyclosome activation by FZY antibodies in *Xenopus* egg extracts may merely reflect activation of the spindle assembly checkpoint.

We do not favour this interpretation for the following reasons. Firstly, to reproduce the spindle assembly checkpoint, a high nuclear density is required to arrest the *Xenopus* embryo cell cycle (Minshull et al., 1994); in contrast, the FZY antibodies arrest the embryonic cell cycle at mitosis in the absence of nuclei. Secondly, taxol, which induces an increase in the amount of polymerized tubulin, as suppression of FZY function may possibly do (O’Toole et al., 1997), does not activate the checkpoint that suppresses cyclin degradation in *Xenopus* egg extracts (Minshull et al., 1994). Thirdly, activation of the spindle assembly checkpoint suppresses degradation of cyclin B, but it has no effect on cyclin A degradation (Minshull et al., 1994); in contrast, blocking FZY function suppresses both cyclin A and B degradation, in *Xenopus* (this work) as in *Drosophila* (Dawson et al., 1995; Sigrist et al., 1995). Finally, activation of the spindle assembly checkpoint is mediated by MAP kinase in *Xenopus* egg extracts (Minshull et al., 1994; Abrieu et al., 1996) and requires X-MAD2 protein activity (Chen et al., 1996); in contrast, blocking FZY function blocks degradation of mitotic cyclins in the absence of MAP kinase activation or X-MAD2 protein. We rather favour the view that FZY function is absolutely required for activation of the APC/cyclosome in the normal cell cycle, i.e. when the spindle checkpoint is not activated in response to spindle perturbation, even though it also appears to be a target component in the degradation machinery for the signal transduction pathway of the spindle assembly checkpoint (Kim et al., 1997).

FZY, like CDC27, undergoes MPF-dependent phosphorylation at mitosis. It is not a component of the APC/cyclosome but rather is a component of a distinct high molecular weight complex. The possibility that the FZY complex may interact transiently with the APC/cyclosome, possibly as a consequence of its mitotic phosphorylation, and cause some change in its structure at the metaphase to anaphase transition cannot, however, be ruled out. This may remove an inhibitor from the APC for example, although direct evidence is lacking to support this view. Figure 9 shows a possible model for this interpretation.

Even though CDC27 and other subunits of the APC/cyclosome are phosphorylated in unfertilized *Xenopus* eggs, the cyclin degradation machinery is silent in extracts prepared from them (CSF extracts). At fertilization, CaMKII activation releases the cyclin degradation pathway from inhibition. We found in the present work that FZY is also required for CaMKII to release the cyclin degradation pathway from CSF inhibition and to allow
sister chromatid segregation. In the same way, FZY is required for activation of cyclin degradation by okadaic acid and microcystin, which activate the cyclin degradation pathway independently of Ca"^{2+} in CSF extracts (Lorca et al., 1991). The APC/cyclosome is believed to bind an inhibitor in CSF extracts (Peters et al., 1996). Although this inhibitor has not been identified, it has been shown to require MAPK activity to maintain its effect in metaphase-arrested oocytes (Minshull et al., 1994; Abrieu et al., 1996). The possibility that this inhibitor may also contribute to the prevention of APC/cyclosome activation in interphase cannot be ruled out. Due to MAPK-dependent phosphorylation, its activity would be prolonged in unfertilized eggs or in CSF extracts. In this case, FZY could participate in the removal of the same (putative) inhibitor from APC in both mitotic and meiotic cell cycles. Work is in progress to characterize this inhibitor.

In *Xenopus*, the degradation of mitotic cyclins is turned off soon after fertilization and MPF inactivation, most likely as an immediate consequence of the dephosphorylation of CDC27, and possibly other APC subunits. This allows mitotic cyclins to start accumulating as soon as the first 83 amino acids was named huCDC27 Δ83. The X-fzy1-Δ83N clone was cut with EcoRI and the resulting insert containing the full-length X-Mad2 cDNA was subcloned into the EcoRI cloning site of the pgex KG vector. Another one lacking the first 83 amino acids was named huCDC27Δ83N, cut with HindIII, and the resulting insert was subcloned into the HindIII cloning site of the pgex KG vector.

**Materials and methods**

cDNA cloning of X-FZY

A PCR fragment was amplified from *Xenopus* oocyte cDNA with fizzy family primers SS13 (ggtggagctnnmrcncacnctcyctg) and SS26 (ggtctggnggangctnwythac) (n = a, c, g, or t; y = c or t; k = g or t; r = a or g; w = a or t; h = a, c, or t). The amplified PCR product (693 bp) was subcloned into Blue-Script vector and used as a probe to screen a λgt10 library of stage 6 oocytes (kindly provided by Dr D.A. Melton). A total of 5×10^6 plaques were transferred to duplicate filters and hyridized at 55°C (2× SSC, 0.1% SDS, 5× Denhardts, 100 μg/ml of salmon sperm DNA), to the 32P-labelled random-primed PCR insert. Filters were washed (2× SSC, 0.1% SDS) at 55°C for 2 h. Three rounds of plaque purification were performed. Purification of λ phages from master plate injection, and of λ double-stranded DNA, were performed according to standard cloning procedures. EcoRI digests of DNA were cloned into pBlue-Script KS2. The sequences of different clones revealed identical overlapping sequences. One of them had a complete ORF of 1521 bp encoding a putative 507 amino acid protein (FZY) and a 3' -untranslated region bearing a AATAAA poly(A) signal. This clone was named X-fzy1. Another one lacked the 69 first amino acids: it was named X-fzy1-Δ69N. The X-fzy1-Δ69N clone was cut with EcoRI and blunted. The insert was subcloned into the EcoRI-blunted cloning site of pGEX2T and pMal-p2 prokaryotic expression vectors.

cDNA cloning of huCDC27, X-Mad2 and UbcX

Oligonucleotides huCDC27 5’ (gtagaaggctggccggcgacctagct) and huCDC27 3’ (cctcctgacgcatgcatcatactacatl) were used to amplify the full-length human CDC27 cDNA. The PCR product was subcloned blunt into the EcoRI cloning site of pBlue-Script or EcoRI–SalI into the EcoRI–SalI cloning site of the pgex KG vector. Another one lacking the first 83 amino acids was named huCDC27Δ83N, cut with HindIII, and the resulting insert was subcloned into the HindIII cloning site of the pQE30 prokaryotic expression vector.

Oligonucleotides X-Mad2 5’ (gaatcggkgtcggccagggaccttg) and X-Mad2 3’ (ctatataagtagaagtagcctg) were used to amplify the full-length Xenopus cDNA. The PCR product was subcloned blunt into the Eco/RI cloning site of pBlue-Script. The pBlue-Script-X-Mad 2 was cut with EcoRI and the resulting insert containing the full-length X-Mad2 cDNA was subcloned into the EcoRI cloning site of pMal-p2 and pgex 2T prokaryotic expression vectors.

Oligonucleotides UbC3 5’ (accataagccgctcagctgacta) and UbC3 3’ (gtctgaagctgatttattagcct) were used to amplify the full-length Xenopus UbcX cDNA. The PCR product was subcloned blunt into the Eco/RI cloning site of pBlue-Script or into the Smal cloning site of pQE30 vector (QIA express type IV construct Quiagen manufacturer).

**Immunization procedures and antibodies**

Fusion proteins of GST with *Xenopus* FZYΔ69N, MAD2 and human CDC27 were expressed in E.coli. Inclusion bodies were prepared and loaded onto SDS-PAGE according to standard procedures. Fusion proteins were electroeluted and used to immunize rabbits. Immune sera directed against *Xenopus* FZY and MAD2 were affinity-purified on immobilized pMal-FZY and pMal-MAD2 fusion proteins, respectively. Immune sera directed against human CDC27 were affinity-purified on immobilized His6-CDC27Δ83N.

Antibodies against *Xenopus* FZR were obtained by immunizing rabbits with the N-terminal peptide (C)DQDYERLLRQVLNQLE (C instead of M for coupling). The peptide was coupled to keyhole limpet haemocyanin (KLH) using m-maleimidobenzoyl-N-hydroxysuccinimide ester. For affinity purification, the peptide was immobilized on sulfo-link agarose beads (Pierce).
To prepare antibodies against the N-terminal 15 amino acid sequence of X-FZY, MAQFAFEDINILSKC (C was added for coupling), the peptide was coupled to thyroblobulin using m-maleimidobenzoyl-N-hydroxysulfosuccinimide (Pierce) for immunization and to bovine serum albumin for affinity purification of antibodies. Phospho-specific MAPK antibody was provided by New England Biolabs.

**In vitro production of [³⁵S]methionine-labelled proteins**

mRNAs encoding full-length human cyclin B1, Xenopus FZY and FZR, as well as rat brain CaMII mutant ending at Lys290, were transcribed in vitro from the corresponding clones with either T7 or T3 RNA polymerases. The mRNAs were translated in a rabbit reticulocyte lysate in the presence of [³⁵S]methionine except for CaMII.

**In vitro degradation and ubiquitylation assays**

For degradation assays, 1 μl of cyclin B1 was incubated at room temperature with 20 μl of interphase, mitotic or CSF egg extracts containing 2 μg of affinity-purified FZY antibodies or the same amount of rabbit immunoglobulins. For ubiquitylation assays, 1.5 μl of in vitro translated cyclin B1 was incubated for 15 min at room temperature with 10 μl of protein A-Sepharose beads containing APC/cyclosome immunopurified from 15 μl of extract and 5 μl of reaction mixture containing 1 mg/ml bovine ubiquitin (Sigma), 0.5 μg of Xenopus ubiquitin-activating enzyme E1, 0.5 μg of Xenopus UbcX, 10 nM Tris–HCl pH 7.5, 100 mM KCl, 2 mM MgCl₂, and a ATP-regenerating cocktail. Reactions were stopped by adding Laemmli buffer and were analysed by SDS–PAGE.

**Xenopus egg extracts**

CSF extracts were prepared from unfertilized Xenopus eggs as described by Murray and Kirschner (1989). To prepare interphase extracts, dejellied CSF extracts were prepared from unfertilized Xenopus egg extracts containing 2 μg of affinity-purified FZY antibodies or the same amount of rabbit immunoglobulins. For ubiquitylation assays, 1.5 μl of in vitro translated cyclin B1 was incubated for 15 min at room temperature with 10 μl of protein A-Sepharose beads containing APC/cyclosome immunopurified from 15 μl of extract and 5 μl of reaction mixture containing 1 mg/ml bovine ubiquitin (Sigma), 0.5 μg of Xenopus ubiquitin-activating enzyme E1, 0.5 μg of Xenopus UbcX, 10 nM Tris–HCl pH 7.5, 100 mM KCl, 2 mM MgCl₂, and a ATP-regenerating cocktail. Reactions were stopped by adding Laemmli buffer and were analysed by SDS–PAGE.

**Immunoprecipitation**

Twenty μl of extracts were diluted with 1 ml of buffer containing 10 mM NaH₂PO₄, 100 mM NaCl, 50 mM NaF, 50 mM KCl, 0.25 mM MgCl₂, 0.5 mM CaCl₂, 0.025 mM Na EGTA, 1.25 mM Hepes–NaOH pH 7.7) and the eggs were activated electrically. Extracts were prepared as above 40 min after activation and stored at ~80°C.

**Nuclear and spindle assembly and return to interphase**

CSF-arrested extracts were obtained as previously described (Morin et al., 1994). To induce the formation of interphase nuclei and DNA replication, permeabilized Xenopus sperm nuclei (200 μl) were added to CSF-arrested extracts previously treated with CaCl₂ (0.4 mM). After 90 min, 0.5 vol. of untreated CSF-arrested extract was added to trigger nuclear envelope breakdown. Subsequently, the mix was supplemented with rhodamine-labelled tubulin (20 μg/ml). Spindle assembly was followed by observation with a fluorescence microscope using Hoechst 33342 to stain chromosomes and rhodamine–tubulin fluorescence to visualize microtubules. Once metaphase spindles were assembled, 0.1 μg/ml of affinity-purified FZY antibodies or the same amount of rabbit immunoglobulins were added. After 15 min, the constitutively active in vitro translated CaMII was added (0.1 vol.) to induce return to interphase.

**Acknowledgements**

We thank Jean Derancourt and Simon Galas for help with computer programs, Danielle Casanova and Sandrine Lacardeur for rabbit immunization, Claude Cavadore for her assistance, Martine Peytavin for secretarial assistance, François Martin for providing E1 protein and Daniel Fisher for critical comments of the manuscript. This work was supported by grants from the ‘Association pour la Recherche sur le Cancer’ and the ‘Ligue Nationale Contre le Cancer’ to J.C.L. and T.L. A.C. is a post-doctoral fellow supported by ‘Beca de Formacion de Personal Investigador en el Extranjero’, Ministerio de Educacion y Ciencia and ‘Training and Mobility of Researchers’, European Commission.

**References**


Received December 29, 1997; revised March 25, 1998; accepted April 30, 1998

**Role of Fizzy in APC/cyclosome activation in *Xenopus***