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

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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 transesterified 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 (Goebl 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).

salivary gland endoreplication (Sigrist and Lehner, 1997). *Drosophila FZY* shares sequence similarity with budding yeast CDC20 and fission yeast SLP1, whose inactivation also suppresses entry into anaphase (Hartwell and Smith, 1985; Sethi *et al.*, 1991; Guacci *et al.*, 1994; Matsumoto, 1997). Apart from CDC20, a protein more closely related to FZR, HCT-1/CDH1 has been shown to be required for the degradation of the mitotic cyclin CLB2 in G₁ (Schwab *et al.*, 1997; Visintin *et al.*, 1997).

In the present work, we have characterized the *Xenopus* homologue of *Drosophila FZY*, and shown that it is part of a high molecular weight complex distinct from the APC/cyclosome. Using an antibody which blocks FZY function, we have shown that, even though not required for phosphorylation of CDC27 and other APC subunits induced by cyclin B-cdc2 kinase, FZY is absolutely required for APC/cyclosome activation, degradation of mitotic cyclins A and B, and chromatid segregation in cell cycle extracts prepared from *Xenopus* eggs.

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 eggs.

The electrophoretic mobility of endogenous FZY decreased upon addition of cyclin B-cdc2 kinase to an

Fig. 2. X-FZY is expressed and undergoes cell cycle-dependent phosphorylation during the first mitotic *Xenopus* cell cycle. (A) X-FZY is detected by Western blotting in extracts prepared from unfertilized eggs, whereas X-FZR is not detected. Lane 1, extract prepared from a CSF extract (75 μ g of proteins); lane 2, reticulocyte lysate (2 μ l) programmed with X-FZY (estimated amount of 35 S-labelled X-FZY: 0.4 pmol); lane 3, reticulocyte lysate (2 μ l) programmed with X-FZR (estimated amount of 35 S-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. I, 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 μ l into 20 μ l; volume-specific activity: 200 pmol P transferred to H1 histone/min/ μ l); CSF, CSF extract; CSF + CaMKII, the same CSF extract after 45 min incubation with *in vitro* translated, constitutively active CaMKII (2 μ l reticulocyte lysate into 20 μ l CSF extracts). (C) 32 P-incorporation into X-FZY and co-immunoprecipitated proteins after incubation of an interphase extract with highly purified starfish cyclin B-cdc2 kinase. The same experiment as in (B), but [γ - 32 P]ATP (50 μ Ci) was added simultaneously with the kinase. FZY and co-immunoprecipitated proteins were analysed by autoradiography after immunoprecipitation with affinity-purified FZY antibodies.

interphase egg extract (Figure 2B), to migrate then with the same mobility as observed in an extract prepared from metaphase II-arrested eggs (CSF extract). FZY mobility returned to its interphase level when constitutively active calmodulin-dependent protein kinase II (CaMKII) (Lorca *et al.*, 1993) was added to a CSF extract, or when the material precipitated from a CSF extract with affinity-purified antibodies was treated with bovine alkaline phosphatase (not shown). This suggested that FZY function may be controlled by mitotic phosphorylation. Mitotic phosphorylation of FZY was confirmed by monitoring incorporation of 32 P into the protein upon addition of cyclin B-cdc2 kinase to an interphase extract (Figure 2C). In addition to FZY, an as yet unidentified 94 kDa protein was strongly phosphorylated in FZY immunoprecipitates.

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 35 S-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 μ M 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 μ l 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 μ l immunoprecipitated without dilution with affinity-purified CDC27 (IP CDC27) or FZY (IP FZY) antibodies cross-linked to protein A–Sephareose. 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 μ l) 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 μ g) of rabbit immunoglobulins (CT: control). Constitutively active, *in vitro* translated CaMKII (CaMK) was added (2 μ l). 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 μ m (same magnification for all micrographs).

Fig. 5. Antibodies directed against *Xenopus* FZY (FZYAb) suppress degradation of ^{35}S -labelled cyclin B1 triggered by CaMKII or microcystin in CSF extracts. (A and D) A CSF extract (20 μl) received either 2 μg of affinity-purified FZY antibodies or the same amount of rabbit immunoglobulins (Mock Ab), as well as 1 μl of *in vitro* translated ^{35}S -labelled cyclin B1. Ten minutes later, either 2 μl of constitutively active unlabelled CaMKII, *in vitro* translated in reticulocyte lysate (A) or 1 μM microcystin (D) was added. Samples (3 μl) were taken 0.5, 15 and 40 min later and analysed by SDS-PAGE and autoradiography for degradation of cyclin B1. (C and D) Aliquots (40 μl) of a CSF extract were depleted with affinity-purified antibodies immobilized on protein A-Sepharose beads (FZY, antibodies against the N-terminally truncated recombinant protein; N-TER FZY, antibodies against a N-terminal peptide of FZY; see Materials and methods) or mock depleted with the same amount (10 μg) of rabbit immunoglobulins (MOCK). Supernatants (20 μl) were taken and constitutively active *in vitro* translated CaMKII was added (2 μl), as well as 1 μl of *in vitro* translated ^{35}S -labelled cyclin B. Samples (3 μl) were taken 0, 5, 15 and 40 min later and analysed by SDS-PAGE (B). Supernatants (1 μl) were also analysed by Western blotting with affinity-purified FZY antibodies (C) to confirm depletion of the FZY protein.

degradation in CSF extracts containing FZY antibodies. 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 (Abrieu *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 ^{35}S -labelled cyclin B1 in the presence of E1 and UBC-X was assayed. As shown in Figure 6C, APC/cyclosome immunoprecipitated from a control egg extract to which mock antibodies were added prior to cyclin B-cdc2 kinase readily generated polyubiquitinated 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 ^{32}P 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 μ l), prepared 40 min after parthenogenetic activation from *Xenopus* eggs, received either 2 μ g 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 μ l; volumic activity: 200 pmol P/min/ μ l). 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, excepted 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 35 S-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 μ l) containing 2 μ g of FZY antibodies (+) or 2 μ g of control immunoglobulins (–), but starfish cyclin B–cdc2 kinase was added simultaneously with [γ - 32 P]ATP (50 μ Ci). 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

Fig. 7. Differential effects of FZY antibodies added to interphase egg extracts before or after activation of the APC/cyclosome by GST–cyclin B–cdc2 kinase. **(A)** Four μ g of affinity-purified FZY antibodies (FZY Ab) were added to 40 μ l of interphase egg extract. After 10 min, recombinant GST–cyclin B (1 μ g) was added, and a further 60 min later 35 S-radiolabelled cyclin B1, translated in reticulocyte lysate (2 μ l), was added (the same result was obtained if GST–cyclin B and FZY antibodies were added simultaneously). **(B and C)** GST–cyclin B was added to 40 μ l of the same interphase egg extract. After 60 min, 4 μ g of control immunoglobulins (Mock Ab) and affinity-purified FZY antibodies were added (B and C respectively). In all cases, samples (3 μ l) were taken at 0.5, 30 and 60 min after addition of 35 S-labelled cyclin B1 and analysed by SDS–PAGE and autoradiography for degradation of [35 S]cyclin B1 (upper panel), and an additional sample (15 μ l) was taken at 15 min and used for ubiquitinylation assay (lower panel) as described in Materials and methods.

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

Fig. 8. X-MAD2 is not required for the block of cyclin degradation induced by FZY antibodies. **(A)** A CSF extract (100 μ l) was depleted with affinity-purified X-MAD2 antibodies (10 μ g) immobilized on protein A-Sepharose beads. One μ l of supernatant (Depleted CSF) or of the starting extract (CSF) was analysed by Western blotting and probed with affinity-purified X-MAD2 (upper panel) or FZY (lower panel) antibodies. **(B)** The X-MAD2-depleted extract (20 μ l) was treated with 2 μ g of affinity-purified FZY antibodies (FZY Ab) or control immunoglobulins (Mock Ab) and received 1 μ l of *in vitro* translated 35 S-labelled cyclin B1. After 10 min, 1 μ M microcystin was added. Samples (3 μ l) were taken at the indicated times and analysed by SDS-PAGE and autoradiography for degradation of cyclin B1. **(C)** Twenty μ l of CSF extract were treated with 2 μ g of affinity-purified FZY antibodies (FZY Ab) or control immunoglobulins (Mock Ab). After 10 min, antibodies or immunoglobulins were removed with protein A-Sepharose beads, and supernatants immunoprecipitated with affinity-purified CDC27 antibodies cross-linked to protein A-Sepharose beads (IP CDC27). Material eluted from each matrix, as well as 2 μ l of starting material, were analysed by Western blotting and probed with affinity-purified X-MAD2 antibodies.

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/Slp1 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

Fig. 9. A speculative model for the role of FZY in the activation of the APC/cyclosome. The model is discussed in detail in the text.

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 15 min after fertilization, i.e. ~25 min before the first round of DNA replication. In contrast, in budding yeast (Schwab *et al.*, 1997), mammalian cells in culture (Brandeis and Hunt, 1996) and post-mitotic *Drosophila* cells (Sigrist and Lehner, 1997), the activity of the APC/cyclosome is not turned off at the end of mitosis, and newly synthesized mitotic cyclins still undergo degradation in G_1 . The failure to maintain the cyclin degradation pathway active after MPF inactivation in the early *Xenopus* embryo may be due to the lack of expression of FZR. Indeed, FZR has been shown to be required for degradation of mitotic cyclins at interphase in *Drosophila*, and its loss causes progression through an extra division cycle in the epidermis and inhibition of endoreplication in the salivary gland (Sigrist and Lehner, 1997), most likely due to suppression of mitotic cyclin degradation in interphase. In budding yeast also, the Fizzy-related HCT-1 is required

to maintain degradation of the major mitotic cyclin CLB2 in G_1 (Schwab *et al.*, 1997; Visintin *et al.*, 1997). Although their mechanism of action have not been investigated at the molecular level, Fizzy-related proteins may be required to prevent the APC from binding an inhibitory component after the drop in MPF activity and CDC27 dephosphorylation. In this case, both Fizzy and Fizzy-related proteins may possibly regulate the interaction of APC with one (or several) unidentified inhibitory component(s) in a cell cycle-regulated fashion.

Materials and methods

cDNA cloning of X-FZY1

A PCR fragment was amplified from *Xenopus* ovary cDNA with fizzy family primers SS13 (ggcggatcnyknarcrcanacyctygt) and SS26 (ggctctggngngancgntwyathcc) (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 λ gt10 library of stage 6 oocytes (kindly provided by Dr D.A.Melton). A total of 5×10^5 plaques were transferred to duplicate filters and hybridized at 55°C ($2 \times \text{SSC}$, 0.1% SDS, $5 \times \text{Denhardt's}$, 100 $\mu\text{g/ml}$ of salmon sperm DNA), to the ^{32}P -labelled random-primed PCR insert. Filters were washed ($2 \times \text{SSC}$, 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- Δ 69N. The X-fzy1- Δ 69N 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' (gatgacgtgctgcaggaccgcgc) and huCDC27 3' (cccgtcgaccgtcagcactagtcacacatcc) were used to amplify the full-length human CDC27 cDNA. The PCR product was subcloned blunt into the *EcoRV* 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 huCDC27 Δ 83N, cut with *HindIII*, and the resulting insert was subcloned into the *HindIII* cloning site of the pQE30 prokaryotic expression vector.

Oligonucleotides X-Mad2 5' (gaattcatggcgggcagctaacacgtg) and X-Mad2 3' (ctataaaagtaagatcgtagg) were used to amplify the full-length *Xenopus* cDNA. The PCR product was subcloned blunt into the *EcoRV* 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 UbcX 5' (acaatgcttccagattg) and UbcX 3' (ggatcagattctttttctcg) were used to amplify the full-length *Xenopus* UbcX cDNA. The PCR product was subcloned blunt into the *EcoRV* cloning site of pBlue-Script or into the *SmaI* cloning site of pQE30 vector (QIA express type IV construct Qiagen manufacturer).

Immunization procedures and antibodies

Fusion proteins of GST with *Xenopus* FZY Δ 69N, 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 (His)6-CDC27- Δ 83N.

Antibodies against *Xenopus* FZR were obtained by immunizing rabbits with the N-terminal peptide (C)DQDYERRLLRQVNLQNE (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 sulfolink agarose beads (Pierce).

To prepare antibodies against the N-terminal 15 amino acid sequence of X-FZY, MAQFAFETDINSILKC (C was added for coupling), the peptide was coupled to thyrolobulin 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 CaMKII mutant ending at Leu290, 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 CaMKII.

In vitro degradation and ubiquitinylation 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 ubiquitinylation 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 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM MgCl₂ and an 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 unfertilized eggs were transferred in MMR/4 (25 mM NaCl, 0.5 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.

Immunoprecipitation

Twenty µl of extracts were diluted with 1 ml of buffer containing 10 mM NaH₂PO₄, 100 mM NaCl, 50 mM NaF, 80 mM Na β-glycerophosphate, 5 mM EDTA, 0.5% Na deoxycholate, 1% Triton X-100 (IP buffer) and clarified by centrifugation at 10 000 g for 5 min at 4°C. Then 2 µg of affinity-purified antibodies covalently bound or not to protein A-Sepharose beads were added. Immunoprecipitates were recovered by standard methods using protein A-Sepharose beads, washed three times with IP buffer and twice with 50 mM Tris pH 7.5. In the ³²P-labelling experiments, the 20 µl of extracts were first diluted twice with 50 mM Tris pH 7.5, 20 mM EDTA, 2 µM microcystin then diluted with 1 ml of IP buffer supplemented with 0.1% SDS. This buffer was also used during the washing steps.

Gel filtration

A CSF extract was diluted twice with 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol (DTT) (buffer A) and clarified by centrifugation at 4°C for 20 min at 50 000 r.p.m. in the TLA 100.4 rotor of a Beckman centrifuge. After buffer exchanging on a desalting PD10 column (Pharmacia) equilibrated with IP buffer, the supernatant was filtered through a 0.45 µm Millipore membrane and applied to a Hiload 16/60 Superdex 200 column equilibrated with IP buffer. The flow rate was 1 ml/min, and 2 ml fractions were collected. Calibration of the column was performed using thyroglobulin (670 kDa), catalase (230 kDa) and ovalbumin (43 kDa) as molecular weight markers (Pharmacia).

Cdc2 kinase, E1 and recombinant proteins

Starfish cyclin B-cdc2 kinase (MPF) was prepared as previously described (Labbé *et al.*, 1991). *Xenopus* ubiquitin-activating enzyme E1 was purified from unfertilized eggs as described by Haas and Bright (1988).

To make recombinant UbcX, we tagged the full-length UbcX coding sequence with six histidines at the N-terminus and expressed it in *E. coli* with Qiaexpress vector pQE30 (Qiagen). The protein was purified according to the manufacturer's instructions. GST-cyclin B, which contains an N-terminal fusion of GST to a sea urchin type B cyclin, was expressed in *E. coli* (Abrieu *et al.*, 1996) and affinity purified (Pharmacia).

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 CaMKII was added (0.1 vol.) to induce return to interphase.

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