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Phosphorylation of the Cyclosome Is Required for Its Stimulation by Fizzy/cdc20

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Exit from mitosis in eukaryotic cells is regulated by the cyclosome (also called anaphase promoting complex or APC), a multisubunit ubiquitin ligase that acts on mitotic cyclins. Previous studies in a cell-free system from clam oocytes have shown that the activation of the cyclosome at the end of mitosis involves its phosphorylation by protein kinase Cdk1/cyclin B. Genetic and biochemical studies have furthermore indicated that cyclosome activity also requires a WD-40 repeat containing protein called Fizzy (FZY) or Cdc20. It has been suggested [Fang *et al.* (1998) *Mol. Cell* 2, 163–171] that in the presence of FZY, the phosphorylation of the cyclosome is not critical for its activation. By contrast, we find that the activity of the interphase, non-phosphorylated form of the cyclosome from clam embryos is not stimulated by FZY to a significant extent. However, when interphase cyclosome is first incubated with protein kinase Cdk1/cyclin B, the subsequent supplementation of FZY greatly stimulates its cyclin-ubiquitin ligase activity. Furthermore, phosphatase treatment of purified mitotic cyclosome prevents its stimulation by FZY, a process that can be reversed by the action of protein kinase Cdk1/cyclin B. We conclude that in the early embryonic cell cycles, the primary event in the activation of the cyclosome at the end of mitosis is its Cdk1-dependent phosphorylation and activation by FZY takes place in a subsequent process.

Exit from mitosis in eukaryotic cells requires the ubiquitin-dependent degradation of cell cycle regulatory proteins, such as mitotic cyclins and anaphase inhibitors (reviewed in 1, 2). The degradation of these proteins is controlled by a multi-subunit ubiquitin ligase known as the cyclosome (3) or anaphase-promoting complex (APC) (4). In early embryonic cell cycles, the cyclosome is inactive in the interphase, but becomes active at the end of mitosis. We have been studying the mechanisms of the regulation of cyclosome activity in a cell-free system from clam oocyctes. The inactive, interphase form of the cyclosome can be converted *in vitro* to the active form by incubation with protein kinase Cdk1/cyclin B (3, 5), and the mitotic form can be inactivated by treatment with a protein phosphatase (6). We have concluded that in the early embryonic cell cycles, the activity of the cyclosome is regulated by reversible phosphorylation (6). The mitotic, phosphorylated form of the cyclosome can be purified by affinity chromatography on immobilized Suc1 (7), an essential cell cycle protein that has high affinity to Cdk1 and to some phosphorylated proteins. In the course of that study we have noted that the cyclin-ubiquitin ligase activity of the cyclosome decreased markedly following affinity purification, but activity could be restored by the addition of the flow-through fraction that was not adsorbed to the affinity column (7). These findings indicated the involvement of additional factor(s) in the regulation of cyclosome activity.

Additional regulators of cyclosome activity have been discovered by genetic studies. In *Drosophila melanogaster,* two WD-40 repeat containing proteins, *fizzy* (FZY) and *fizzy-related* (FZR), have been shown to be required for the degradation of mitotic cyclins in mitosis and in the G1 phase of the cell cycle, respectively (8, 9). Similar, though not identical functions have been described for two homologous proteins in *Saccharomyces cerevisiae,* Cdc20 and Cdh1/Hct1, respectively (10, 11). The function of FZR/Cdh1 appears to be restricted to somatic and yeast cell cycles, where it keeps the cyclosome active during the G1 phase (9, 12–15). By contrast, early embryonic cell cycles have no G1 phase, and consist of rapidly alternating S and M phases. Accordingly, only FZY (and not FZR) is found in early embryonic cells of *Drosophila* (9) and

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Xenopus (16), while FZR is expressed only at a later stage of embryonic development, when cell cycles are switched to the somatic mode. Therefore, when considering the mode of the regulation of cyclosome activity in early embryonic systems, such as clam oocytes or *Xenopus* eggs, only the effects of FZY have to be taken into account.

This study was initiated in order to examine whether FZY can functionally replace the cyclosome-stimulatory factor in the flow-through fraction, and if so, to characterize the mode of action of FZY in this biochemical system. In the course of this work, two recent papers have reported that FZY stimulates cyclosome activity *in vitro*. Lorca and co-workers (16) have reported that antibodies directed against *Xenopus* FZY block the degradation and ubiquitinylation of cyclin B in extracts of mitotic *Xenopus* eggs. Fang *et al.* (17) have reported that human FZY/Cdc20, translated *in vitro* in reticulocyte lysates, binds to immunopurified cyclosome from *Xenopus* eggs and stimulates its activity. These authors have furthermore reported that FZY stimulates the activity of both mitotic (phosphorylated) and interphase (non-phosphorylated) preparations of cyclosome from Xenopus eggs. It was concluded that the binding of FZY/Cdc20 to the cyclosome/APC, and not the phosphorylation of the cyclosome/APC, is the critical step in the activation of the cyclosome/APC (17). This suggestion is at variance with our earlier findings that the phosphorylation of the cyclosome from early embryonic cells is required for its activity (6), and that the fraction not adsorbed to Suc1-Sepharose stimulates the activity of the phosphorylated, but not of the non-phosphorylated form of the cyclosome (7). We have therefore re-examined this problem and our results are reported in the present paper. We discuss possible reasons for the difference between our results and those of Fang *et al.* (17).

MATERIALS AND METHODS

Extracts of M-phase clam oocytes and interphase two-cell clam embryos were prepared as described previously (5, 18). The mitotic form of the cyclosome was purified by affinity chromatography on Suc1-Sepharose (7). The flow-through fraction of this procedure was prepared as described (7). Cyclosome from interphase embryos was partially purified by chromatography on DEAE-cellulose, salt extraction and gel filtration on Superose-6, as described (19). Cdk1/GST-Δ88-cyclin B was prepared and purified by sequential affinity chromatography on Suc1-Sepharose and GSH-agarose, as described previously (7, 19). We have used this preparation for the present study, because it has high kinase activity, but lacks the N-terminal "destruction box" region of cyclin, and therefore does not interfere with the cyclin-ubiquitin ligation assay (7, 19). To facilitate description, this preparation is referred to as Cdk1/cyclin B.

Human FZY cDNA was obtained from EST clone 656-h22, which was sequenced and found to be identical to p55cdc (FZY) cloned by Weinstein *et al.* (20). This cDNA was cloned into the pT7T3 plasmid. Translation of FZY *in vitro* was carried out in a reaction volume of 55 μl containing 50 μl TnT Quick Coupled Transcription-Translation mix (Promega), 1 μg of the above-described plasmid, 50 μCi ^{35}S-methionine and 0.02 mM unlabeled methionine. Following incubation at 30°C for 90 min, the formation of FZY was verified by gel electrophoresis and radioautography. In all preparations, more than 80% of total protein radioactivity was in the 55-kDa FZY protein. Translation mix lacking FZY was prepared in a similar incubation lacking the plasmid. For immunodepletion of Cdc27, a monoclonal antibody directed against human Cdc27 (21) was covalently linked to Affi-Prep Protein A beads (BioRad), as described (22). 40 μl of translation mix containing FZY was mixed with 5 μl of anti-Cdc27-protein A beads (~1.2 μg of IgG/μl beads) at 0°C for 1 h. The beads were removed by centrifugation, the treatment was repeated and the supernatants were filtered through a 0.45-μM Microspin filter.

The cyclin-ubiquitin ligase activity of the cyclosome was determined in a reaction mixture containing in a volume of 10 μl: 40 mM Tris-HCl (pH 7.6), 1 mg/ml reduced-carboxymethylated bovine serum albumin, 1 mM dithiothreitol, 5 mM $MgCl_2$, 10 mM phosphocreatine, 50 μg/ml creatine phosphokinase, 50 μM ubiquitin, 1 μM ubiquitin aldehyde, 1 pmol E1, 5 pmol E2-C, 1 μM okadaic acid, 1-2 pmol (~2×10^5 cpm) of ^{125}I-labeled cyclin B (13-91)/protein A (termed "^{125}I-cyclin" subsequently) and source of cyclosome as specified. The sources of materials used in the reaction mixture are described in ref 7. Following incubation at 18°C for 60 min, samples were subjected to electrophoresis on a 12.5% polyacrylamide/SDS gel. Results were quantified with a phosphorimager.

RESULTS

We have first examined whether FZY stimulates the activity of cyclosome purified from clam oocytes, as does the fraction not adsorbed to the Suc1-Sepharose affinity column (7). Because recombinant FZY expressed in bacteria is not soluble (16, 17), and it is also mostly insoluble when expressed with a baculovirus vector in insect cells (our observations), we have used FZY translated *in vitro* in reticulocyte lysate (17). The addition of increasing amounts of such preparation of FZY to the purified mitotic cyclosome strongly stimulated cyclin-ubiquitin ligase activity (Fig. 1, closed triangles). Because FZY was added in a translation mix containing reticulocyte proteins, we have examined the possible contribution of the translation mix to the observed effect. We found that translation mix lacking FZY did stimulate cyclosome activity (Fig. 1, open triangles), but to a much less extent than did similar amounts of translation mix containing FZY. Without added cyclosome, a low activity of cyclin-ubiquitin ligation could be observed with translation mix containing or lacking FZY (Fig. 1, filled and open circles). It thus seems that reticulocyte lysates contain a low amount of FZY-like activity, and a lower amount of cyclosome-like activity, but most of the stimulation of the activity of cyclosome from mitotic clam oocytes was due to *in vitro*-translated FZY.

We have next examined whether *in vitro*-translated FZY can stimulate the activity of the interphase form of the cyclosome. To prepare interphase cyclosome, we have used extracts of clam embryos arrested by emetine at the interphase of the second cell cycle (5, 18). Since the interphase form of the cyclosome does not bind to Suc1-Sepharose (7), it was partially purified by chromatography on DEAE-cellulose followed by gel fil-

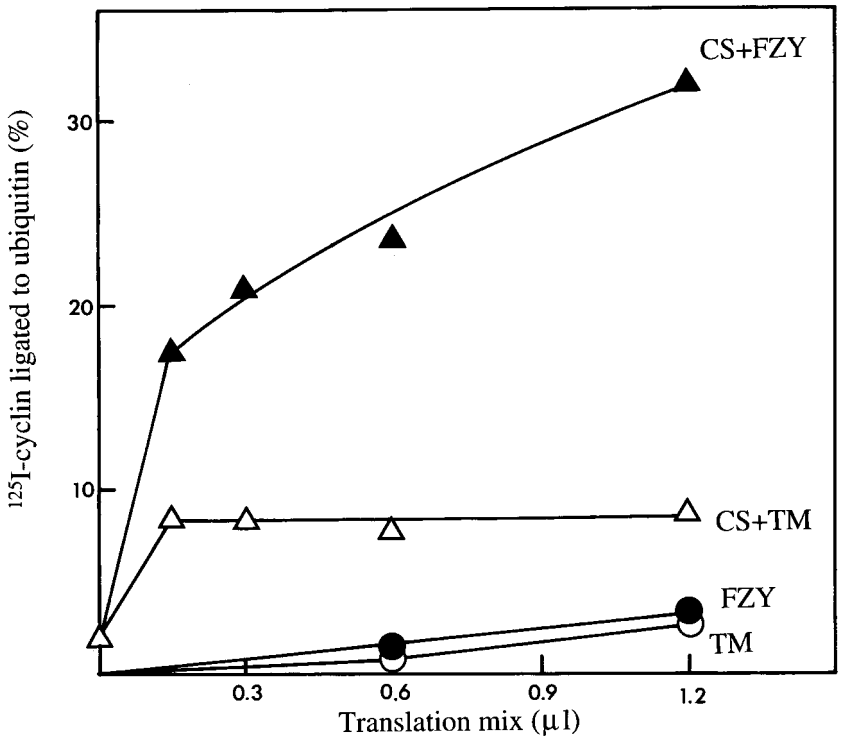

FIG. 1. FZY stimulates the activity of purified cyclosome from mitotic clam oocytes. ^{125}I-cyclin-ubiquitin ligation was determined as described under "Materials and Methods", in the presence of the indicated amounts of *in vitro*-translated FZY, or translation mix lacking FZY ("TM"). Where indicated, 0.5 μl of affinity-purified cyclosome from mitotic clam oocytes ("CS") was added. Triangles, with cyclosome; circles, without cyclosome; filled symbols, with *in vitro*-translated FZY; open symbols, with translation mix lacking FZY.

tration on Superose-6 (19). This preparation of interphase cyclosome had no activity of cyclin-ubiquitin ligation (Fig. 2, lane 2). In accordance with our previous results (7), the addition of the flow-through fraction from the Suc1-Sepharose affinity procedure did not stimulate significantly the activity of the interphase cyclosome (Fig. 2, lane 3). When increasing amounts of *in vitro*-translated FZY were added to the interphase cyclosome preparation, low activity of cyclin-ubiquitin ligation could be seen (Fig. 2, lanes 4 and 5). However, this was entirely due to the cyclin-ubiquitin ligation activity of the translation mix, as shown by the observation that similar amounts of cyclin-ubiquitin conjugates were formed when similar amounts of *in vitro*-translated FZY preparations were incubated in the absence of cyclosome (Fig. 2, lanes 10 and 11). To examine whether the activity of the same preparation of interphase cyclosome can be stimulated by FZY following phosphorylation, interphase cyclosome was first incubated with protein kinase Cdk1/cyclin B in the presence of MgATP, and then protein kinase action was stopped by the addition of staurosporine. Subsequently, the flow-through fraction or increasing amounts of FZY were added and cyclin-ubiquitin ligation activity was determined (Fig. 2, lanes 6–9). Following phosphorylation by the protein kinase, activity was strongly stimulated by the flow-through fraction (Fig. 2, *cf.* lanes 6 and 7) and even more by high concentrations of FZY (Fig. 2, lanes 8 and 9). It is notable that the flow-through fraction stimulated mainly the accumulation of low molecular mass conjugates, while FZY stimulated the accumulation of high molecular mass conjugates, containing long polyubiquitin chains (Fig. 2, lanes 7–9). This may be due to the presence of an enzyme that disassembles polyubiquitin chains in the flow-through fraction, or to a difference between the mode of action of factor(s) present in this fraction and that of FZY. Control experiments indicated that the addition of staurosporine prior to incubation of interphase cyclosome with protein kinase Cdk1/cyclin B completely prevented the stimulation of cyclin-ubiquitin ligation activity by FZY (data not shown). These results suggest that FZY stimulates the activity of the cyclosome only following a phosphorylation reaction carried out by protein kinase Cdk1/cyclin B.

Since the above-described experiments were carried out with partially purified preparation of cyclosome from interphase cells, it was possible that some of the observed effects were due to indirect mechanisms. For example, it could be that the preparation of interphase cyclosome contained an inhibitor of FZY action, and that this inhibitor was inactivated by phosphorylation catalyzed by Cdk1/cyclin B. We have therefore further

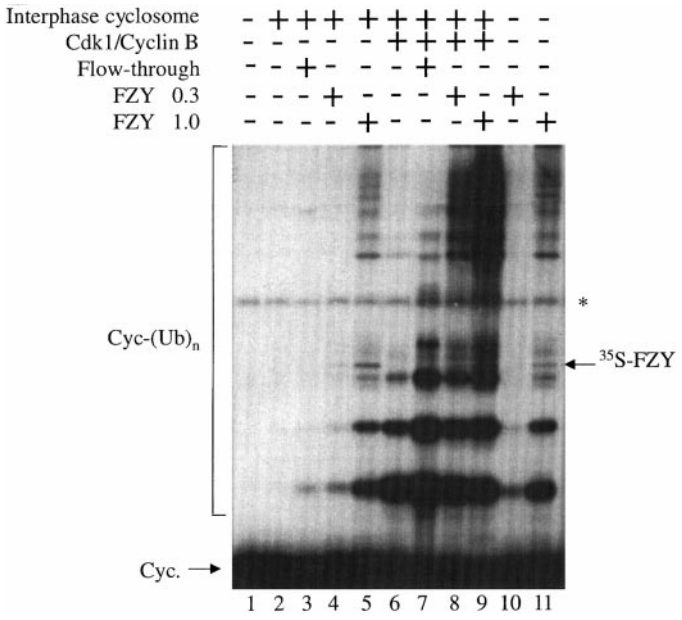

FIG. 2. Activity of cyclosome from interphase clam embryos is stimulated by FZY only following phosphorylation by protein kinase Cdk1/cyclin B. Samples of 1 μl of interphase cyclosome preparation were incubated (18°C, 20 min) with 500 units of Cdk1/cyclin B (lanes 6–9) or without the protein kinase (lanes 2–5), in a reaction mixture similar to that used for the assay of cyclin-ubiquitin ligation, except that ^{125}I-cyclin was omitted. Subsequently, staurosporine (5 μM) was added to all samples, followed by the addition of flow-through fraction (ref. 7, 10 μg of protein) or in vitro-translated FZY (0.3 or 1.0 μl), as indicated. Following the addition of ^{125}I-cyclin, all samples were incubated for a further 60 min for the assay of cyclin-ubiquitin ligation. Cyc., position of free ^{125}I-cyclin; Cyc-(Ub$_n$), cyclin-ubiquitin conjugates. All labeled protein bands of molecular size higher than free cyclin are cyclin-ubiquitin conjugates, except for a contaminating protein labeled by * symbol, and FZY labeled with ^{35}S-methionine ("^{35}S-FZY"), seen in lanes 5 and 11.

examined this problem with the aid of purified mitotic cyclosome that had been subjected to phosphatase treatment. In the experiment shown in Fig. 3, purified cyclosome from mitotic clam oocytes was incubated with lambda phosphatase and then phosphatase action was terminated by the addition of vanadate (23). Control experiments (not shown) have indicated that vanadate effectively inhibited phosphatase action, without affecting the cyclin-ubiquitin ligation reaction. Treatment of purified mitotic cyclosome with lambda phosphatase markedly reduced its cyclin-ubiquitin ligation activity and prevented most of the stimulation by FZY or the flow-through fraction (Fig. 3, compare lanes 4–6 with lanes 1–3). At least a part of the slight activity observed in the incubation of phosphatase-treated cyclosome with FZY (Fig. 3, lane 6) is due to the cyclin-ubiquitin ligation activity of reticulocyte lysate translation mix, supplemented together with *in vitro*-translated FZY. The question arose whether the decrease in cyclin-ubiquitin ligation activity of phosphatase-treated cyclosome is due to its irreversible inactivation, caused by the conditions of incubation of phosphatase treatment. However, we found that most of FZY-stimulated activity could be restored by a further incubation of phosphatase-treated cyclosome with protein kinase Cdk1/cyclin B (Fig. 3, lanes 7–9). The cumulative evidence thus strongly indicates that phosphorylation of the cyclosome is required for its stimulation by FZY.

Our finding that reticulocyte lysate, present in the *in vitro* translation mix, had some cyclin-ubiquitin ligation activity (Fig. 2, lane 11) was surprising, because reticulocytes are terminally differentiating cells that lack nuclei. We have therefore examined whether the cyclin-ubiquitin ligation activity is due to the presence of cyclosomes in reticulocyte lysates. Immunoblotting of reticulocyte lysate with an antibody directed against Cdc27 (a subunit of the cyclosome) indicated the pres-

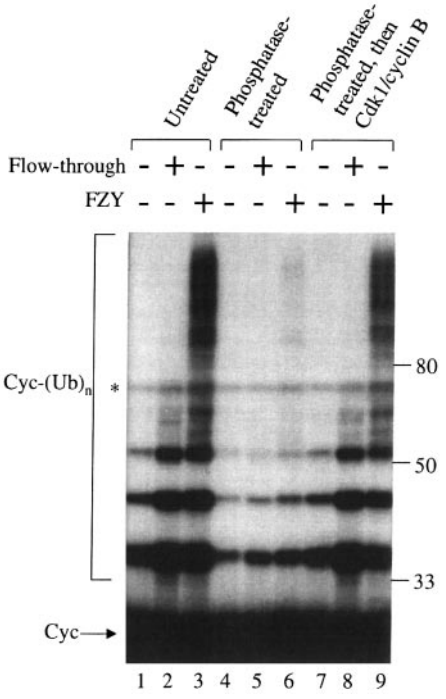

FIG. 3. Phosphatase treatment of the mitotic form of the cyclosome prevents its stimulation by FZY. Samples of 1 μl of affinity-purified mitotic cyclosome preparation were incubated (18°C, 60 min) with 10 units of lambda phosphatase (New England Biolabs) (lanes 4–9), or without phosphatase (lanes 1–3), in a reaction mixture similar to that used for the assay of cyclin-ubiquitin ligation (see "Materials and Methods"), except that ^{125}I-cyclin was omitted and $MnCl_2$ (0.1 mM) was added. Subsequently, 2 mM sodium vanadate was added to all samples, followed by the supplementation of Cdk1/cyclin B (500 units) to the indicated samples. Following a second incubation of 60 min at 18°C, staurosporine (5 μM) was added to all samples. Where indicated, flow-through fraction (ref. 7, 10 μg of protein), or *in vitro*-translated FZY (0.3 μl) were supplemented. ^{125}I-cyclin was added to all samples, and cyclin-ubiquitin ligation was assayed after a third incubation of 60 min. Cyc., position of free cyclin; Cyc(Ub$_n$), cyclin-ubiquitin conjugates; *, contamination in the preparation of labeled cyclin. Numbers on the right side indicate the position of molecular mass protein markers (kDa).

ence of this protein (Fig. 4A). To examine whether Cdc27 is a part of a functional cyclosome complex in reticulocyte lysates, we have subjected these lysates (containing *in vitro*-translated FZY) to immunodepletion with an anti-Cdc27 antibody. This immunodepletion procedure removed part of the Cdc27 protein (Fig. 4A). This was accompanied by a partial decrease in the cyclin-ubiquitin ligation activity of the FZY translation mix, assayed with large amounts of translation mix to increase the sensitivity of detection (Fig. 4B). We have concluded that at least a part of the cyclin-ubiquitin ligation activity of reticulocyte lysates is due to the presence of functional cyclosomes.

DISCUSSION

Our results indicate that in the early embryonic cell cycles, the primary event in the activation of the cyclosome at the end of mitosis is its Cdk1-dependent phosphorylation. Subsequently, FZY stimulates the activity of the phosphorylated cyclosome. This conclusion is based on the observations that the cyclin-ubiquitin ligase activity of the interphase form of the cyclosome is stimulated by FZY only following phosphorylation by Cdk1/cyclin B (Fig. 2) and that phosphatase treatment of the purified mitotic cyclosome prevents its stimulation by FZY, a process that can be reversed by the action of protein kinase Cdk1/cyclin B (Fig. 3). It should be emphasized that our conclusions are limited to the case of early embryonic cell cycles, and there is no sufficient evidence at present for the involvement of cyclosome phosphorylation in its activation in somatic cell cycles.

Our results are different from those of Fang *et al.* (17), who reported that FZY stimulates the activity of both mitotic and interphase cyclosomes from *Xenopus* eggs and suggested that phosphorylation of cyclosome is not critical for its activation. It does not seem likely that this difference in results is due to species differences in regulatory mechanisms between clams and frogs, because both are early embryonic systems activated by Cdk1/cyclin B (3, 5, 24, 25), and basic cell cycle regulatory mechanisms are strongly conserved in evolution in eukaryotes (1, 2). It rather seems that the discrepancy in results is due to differences in experimental conditions. An important difference is the mode of preparation of interphase extracts. Our method consists of fertilization of clam oocyctes and the addition of the protein synthesis inhibitor emetine at the end of mitosis 1 (5, 18). Under these conditions, mitotic cyclins are degraded and the first cell division takes place, but embryos are arrested in the two-cell state due to the inhibition of new cyclin synthesis. Only batches in which 98–100% of embryos are arrested in the two-cell state are used for the preparation of interphase extracts (18). In such interphase extracts, levels of protein kinase Cdk1/cyclin B activity are less than 1% of those in M-phase extracts (7), and there is virtually no cyclosome activity (Fig. 2). By contrast, *Xenopus* egg interphase extracts are prepared by activation of eggs by electrical stimulation or ionophore treatment, followed by incubation in the presence of a protein synthesis inhibitor (2). Thus, these cells are arrested before mitosis 1, while clam embryos are arrested after mitosis 1. It appears that in the interphase before mitosis 1, the inactivation of the cyclosome is not complete, because in such *Xenopus* interphase extracts, significant residual amounts of cyclosome activity can be seen (14, 17). It seems possible, therefore, that cyclosomes from such preparations of interphase *Xenopus* eggs are partially phosphorylated, which would explain their stimulation by FZY. A further difference is that the results of Fang *et al.* (17) were not corrected

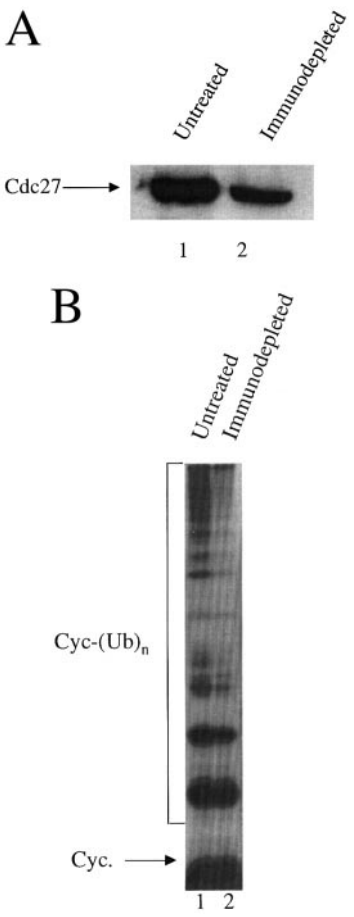

FIG. 4. Presence of cyclosome in reticulocyte lysates. Translation mix containing *in vitro*-translated FZY was subjected to immunodepletion with anti-Cdc27 antibodies, as described under "Materials and Methods". (A) Detection of Cdc27 by immunoblotting. Samples of 70 μg protein (equivalent to 1 μl of untreated translation mix) of untreated or immunodepleted preparations were separated on 10% polyacrylamide-SDS gel and immunoblotted with an anti-Cdc27 antibody (Transduction Laboratories). (B) Cyclosome activity. Cyclin-ubiquitin ligation was assayed in samples of 140 μg of the same preparations as described under "Materials and Methods", except that the temperature of incubation was 30°C.

for cyclosome activity in the translation mix containing *in vitro*-translated FZY. Such correction can be significant if large amounts of translation mix are used (Fig. 4).

The mode of action of FZY and its possible regulation in the cell cycle are not known and require further investigation. In somatic cell cycles, levels of FZY oscillate with a peak in mitosis followed by rapid decline to low levels in G1 (14, 26). By contrast, FZY is present at high levels in both M-phase and interphase Xenopus eggs (16). We have also observed FZY-like cyclosome-stimulating activities in extracts of both M-phase and interphase clam oocytes (unpublished observations). Thus, in early embryonic systems, FZY activity does not seem to be regulated by changes in its levels. Phosphorylation of FZY has been observed in mitosis in both somatic (20, 26) and early embryonic (16) cells. While it has been established that phosphorylation of Cdh1/FZR in yeast negatively regulates its activity (12, 15, 27), the functional significance of the phosphorylation of FZY remains to be elucidated.

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