

Phosphorylation of Cdc20/Fizzy Negatively Regulates the Mammalian Cyclosome/APC in the Mitotic Checkpoint

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The cyclosome/anaphase promoting complex (APC) is a multisubunit ubiquitin ligase that targets mitotic regulators for degradation in exit from mitosis. It is activated at the end of mitosis by phosphorylation and association with the WD-40 protein Cdc20/Fizzy and is then kept active in the G1 phase by association with Cdh1/Hct1. The mitotic checkpoint system that keeps cells with defective spindles from leaving mitosis interacts with Cdc20 and prevents its stimulatory action on the cyclosome. The activity of Cdh1 is negatively regulated by phosphorylation, while the abundance of Cdc20 is cell cycle regulated, with a peak in M-phase. Cdc20 is also phosphorylated in G2/M and in mitotically arrested cells, but the role of phosphorylation remained unknown. Here we show that phosphorylation of Cdc20 by Cdk1/cyclin B abrogates its ability to activate cyclosome/APC from mitotic HeLa cells. A nonphosphorylatable derivative of Cdc20 stimulates cyclin-ubiquitin ligation in extracts from nocodazole-arrested cells to a much greater extent than does wild-type Cdc20. It is suggested that inhibitory phosphorylation of Cdc20/Fizzy may have a role in keeping the cyclosome inactive in early mitosis and under conditions of mitotic checkpoint arrest. © 2000 Academic Press

Recent studies have shown that a multisubunit ubiquitin ligase complex, called the cyclosome (1) or anaphase-promoting complex (APC) (2), has important roles in the control of the cell division cycle. It promotes the degradation of cell cycle regulatory proteins such as mitotic cyclins and inhibitors of anaphase initiation, events necessary for exit from mitosis (reviewed in Refs. 3–5). The cyclosome/APC is inactive in the S-phase and is converted to an active form at the end of mitosis. Activation of the cyclosome requires its phos-

phorylation (6, 7) and its binding to a WD-40 repeat-containing protein called Fizzy or Cdc20 (8–10). Using a cell-free system from clam oocytes that reproduces the early embryonic cell cycles (1), we have previously shown that the phosphorylation of the cyclosome/APC is necessary for its activation by Cdc20/APC (11). In somatic cell cycles, the cyclosome is kept active until the end of the G1 phase by another WD-40 protein called Fizzy-related (12) or Cdh1/Hct1 (13, 14). The activity of Cdh1 is negatively regulated by phosphorylation (15–17), while the abundance of Cdc20 is cell cycle regulated, with a peak in M-phase (18). Cdc20 is also phosphorylated in G2/M (18) and in mitotically arrested cells (10, 19), but the role of this phosphorylation remained unknown.

This study was initiated to examine the mode of the regulation of cyclosome activity in somatic mammalian cells. Somatic cells have the same basic mechanisms that operate in early embryonic cells, but have additional G1 and G2 phases and respond to a variety of extracellular cues. In addition, “checkpoint control” mechanisms, that delay cell cycle progression until the previous stages have been completed, operate much more efficiently in somatic than in early embryonic cells. One of these, the spindle checkpoint system, is activated by unattached kinetochores or damaged spindles and delays exit from mitosis by inhibiting the cyclosome/APC (20–22). It has been shown that the mitotic checkpoint protein Mad2 interacts with Cdc20 and prevents its action to stimulate the cyclosome/APC (23–25). Here we show that phosphorylation of Cdc20, that takes place under conditions of mitotic checkpoint conditions, also abrogates its action to stimulate the cyclosome/APC from mitotic HeLa cells. Thus, the inhibitory phosphorylation of Cdc20 may have a role as a safeguard to keep the cyclosome inactive in early mitosis and under conditions of mitotic checkpoint arrest.

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MATERIALS AND METHODS

Cell culture and synchronization. HeLa S3 cells were grown in suspension culture in DMEM supplemented with 10% fetal bovine serum, at 37°C in the presence of 5% CO₂. Cells were synchronized by double thymidine block, as follows: Logarithmically growing cultures (4–6 × 10⁵ cells/ml) were treated with 2 mM thymidine for 18–24 h, released for 8 h and treated again with thymidine for 18–24 h. S-phase and G1-phase cells were harvested at 2 and 12 h, respectively, following release from the second thymidine block. Cells were arrested in mitosis by treatment with nocodazole (0.2 μg/ml) for 24 h. To prepare “mitotic exit” cells, nocodazole-arrested cells were washed twice with fresh medium, resuspended to 5–7 × 10⁵ cells/ml and cultured at 37°C for further 2 h. In all cases, synchrony was verified by FACScan analysis.

Preparation of extracts and cyclosome purification. All operations were at 0–4°C. 2-L portions of synchronized HeLa cell cultures were collected by centrifugation (400g, 10 min) and were washed twice with phosphate-buffered saline. The cells were resuspended in ~3 volumes of hypotonic buffer [20 mM HEPES-NaOH (pH 7.6), 1.5 mM MgCl₂, 0.5 mM KCl, 1 mM DTT] and were immediately collected by centrifugation. The pellet was resuspended in 1.5–2 volumes of the above hypotonic buffer that contained leupeptin and chymostatin (10 μg/ml, each). The sample was allowed to stay on ice for 30 min and then the cells were disrupted by Dounce homogenization (30 strokes). Following centrifugation at 100,000g for 30 min, the supernatants were collected, mixed with glycerol (10%, final concentration) and stored at –70°C in small samples. Protein concentration of extracts was 8–12 mg/ml.

For partial purification of cyclosomes, extracts from different cell cycle stages (18–20 mg of protein) were separated on a HiLoad 16/60 Superdex 200 prep grade column (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 1 mM dithiothreitol (DTT). Fractions of 2.5 ml were collected at a flow rate of 1.5 ml/min. The fractions were concentrated by ultrafiltration with Centricon-30 (Amicon), diluted 10-fold in a solution consisting of similar to the above buffer, but without NaCl, and concentrated again to a final volume of ~200 μl, followed by the addition of glycerol to 10%. Cyclosome activity was determined in 1-μl samples of column fractions by cyclin-ubiquitin ligation assay (see below), in the presence of 0.5 μl of cyclosome-depleted Cdh1. The peak of cyclosome activity (usually, fractions 17–19) was pooled, divided to small samples and stored at –70°C.

Preparation of Cdh1, Cdc20, and Cdc20 (7xA) mutant. Human Cdc20/Fizzy and Cdh1/Fizzy-related were cloned into a pT7T3 vector as described previously (11, 17). Cdc20 (7xA) mutant was prepared by the QuickChange (Stratagene) PCR based mutagenesis method. The serine residues at positions 41, 408, and 452, as well as the threonine residues at positions 55, 59, 70, and 157 were changed to alanines. A full-length sequence of Cdc20 (7xA) confirmed the introduction of these mutants, as well as the absence of any non-intentional ones. Since recombinant Cdc20 and Cdh1 and derivatives expressed in bacteria or in baculovirus-infected insect cells are all insoluble, they were obtained by *in vitro* translation in reticulocyte lysates, as described previously for Cdc20 (11). As noted previously (11), reticulocyte lysates contain significant amounts of cyclosomes, which are activated by the *in vitro* translated WD-40 proteins, most markedly by Cdh1. Therefore, cyclosomes were removed from all preparations by immunodepletion with anti-Cdc27-Protein A beads, as described (11). Completeness of cyclosome removal was ascertained by immunoblotting with anti-Cdc27, as well as by the decrease of endogenous cyclin-ubiquitin ligation activity to negligible levels.

Assay of cyclin-ubiquitin ligation. The reaction mixture contained in a volume of 10 μl: 40 mM Tris-HCl (pH 7.6), 1 mg/ml carboxymethylated bovine serum albumin, 1 mM DTT, 5 mM MgCl₂, 10 mM phosphocreatine, 50 μg/ml creatine phosphokinase, 0.5 mM ATP, 50 μM ubiquitin, 1 μM ubiquitin aldehyde, 1 pmol E1, 5 pmol E2-C, 1 μM okadaic acid, 1–2 pmol (~1–2 × 10⁵ cpm) of ¹²⁵I-labeled

cyclin B/protein A (termed “¹²⁵I-cyclin”) and cyclosome preparation or crude extract in the linear range of the assay (usually, 0.2–1 μl of cyclosome preparation). Following incubation at 30°C for 60 min, samples were subjected to electrophoresis on a 12.5% polyacrylamide gel. Results were quantified with a phosphorimager.

Binding of ³⁵S-labeled WD-40 proteins to cyclosome/APC. To estimate the binding of WD-40 proteins to cyclosome/APC, ³⁵S-labeled proteins were prepared as described above, except that unlabeled methionine was omitted in *in vitro* translation and preparations were not subjected to cyclosome immunodepletion. Following appropriate incubations (see Fig. 3), the binding of ³⁵S-labeled WD-40 proteins to endogenous reticulocyte cyclosomes was estimated by mixing with 5 μl of anti-Cdc27-Protein A beads (prepared as described in Ref. 11), for 2 h at 4°C. The beads were washed 4 times with 1-ml portions of a buffer that contained 50 mM Tris-HCl (pH 7.6), 150 mM NaCl and 1% NP-40, bound proteins were eluted with SDS sample buffer and were separated on a 10% gel. Controls with Protein A beads bound to non-immune IgG showed no significant non-specific adsorption of all ³⁵S-labeled WD40 proteins used.

RESULTS AND DISCUSSION

Differential effects of Cdc20 and Cdh1 on the activity of mammalian cyclosomes from different stages of the cell cycle. We have first examined whether the cell cycle stage-specific actions of Cdc20/Fizzy and Cdh1, observed in intact cells (8, 12–14), can be reproduced in enzyme preparations from mammalian cells. For this purpose, we prepared extracts from HeLa cells synchronized at different stages of the cell cycle, partially purified cyclosomes from each cell cycle stage by gel filtration chromatography, and examined the effects of Cdc20 and Cdh1 on their cyclin-ubiquitin ligase activity *in vitro*. The preparations of Cdc20 and Cdh1 used for these experiments were obtained by *in vitro* translation in reticulocyte lysates, because these proteins are not soluble when expressed in bacteria or in baculovirus-infected insect cells (11). As shown in Fig. 1a, cyclosomes purified from all cell cycle stages had very low activity without added activators. The addition of Cdh1 markedly stimulated the activity of cyclosomes from all stages of the cell cycle. By contrast, Cdc20 specifically stimulated the activity of cyclosomes derived from “M-phase” (arrested with nocodazole) or “M-phase exit” (shortly after release from nocodazole arrest) cells, but not of those from G1 or S-phase cells. The slight stimulation of cyclosome activity by Cdc20 observed in the latter cases did not exceed that obtained with a similar amount of reticulocyte lysate. It is notable that the activity of cyclosomes from M-phase and M-phase exit cells was similar in the presence of Cdc20 (Fig. 1a), while in crude extracts from the same cells, cyclin-ubiquitin ligation activity was increased greatly in transition from M-phase to M-phase exit (Fig. 1b). This finding indicates that inhibitors as well as activators that affect cyclosome activity are effectively removed by the purification procedure. We have concluded that the cell cycle stage-specific action of Cdc20, that stimulates the degradation of mitotic regulators during exit from mitosis, could be reproduced

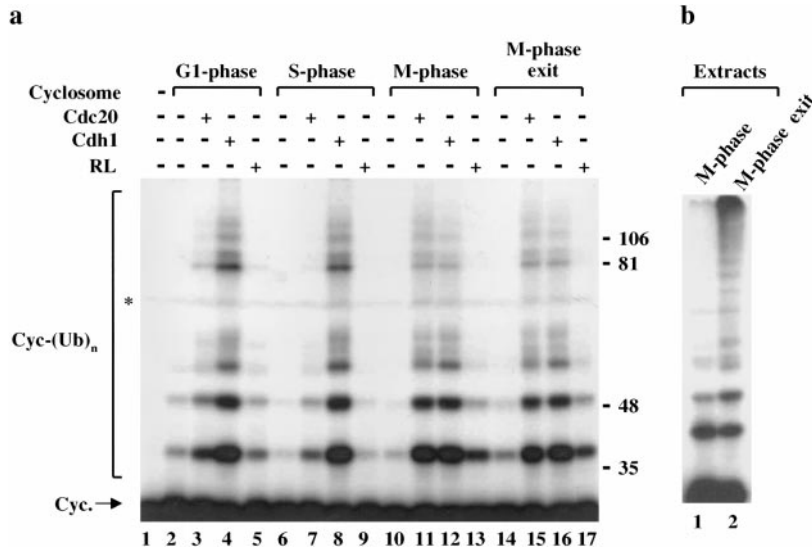


FIG. 1. Differential effects of Cdc20 and Cdh1 on the activity of mammalian cyclosomes from different stages of the cell cycle. (a) Cyclin-ubiquitin ligase activity of cyclosomes from different cell cycle stages was assayed (see Materials and Methods) in the presence of 0.5 μ l of Cdh1, Cdc20 or similarly treated reticulocyte lysate ("RL"), as indicated. (b) Cyclin-ubiquitin ligation was assayed with crude extracts (15 μ g of protein) from the indicated cell cycle stage. Cyc., free 125 I-cyclin; Cyc-(Ub)_n, cyclin-ubiquitin conjugates; *, contamination in the preparation of labeled cyclin. Numbers on the right side of (a) indicate the migration position of molecular mass marker proteins (kDa).

in vitro with cyclosome preparations from mammalian cells.

Phosphorylation of mammalian cyclosome is required for its stimulation by Cdc20. We have next asked whether the M-phase-specific action of Cdc20 on mammalian cyclosomes is due to a requirement for cyclosome phosphorylation, as was previously shown in the early embryonic clam oocyte system (11). This possibility was consistent with the observations that several cyclosome subunits are phosphorylated in mitosis (5), and are still significantly phosphorylated in our M-phase exit preparations (see below). The experiments shown in Fig. 2 indicate that this is indeed the case. Treatment of cyclosomes from M-phase exit cells with lambda phosphatase drastically reduced the stimulation of activity by Cdc20 (Fig. 2a, compare lanes 2 and 5). On the other hand, the stimulatory action of Cdh1 was only slightly reduced by phosphatase treatment (Fig. 2a, lanes 3 and 6). Similar findings were obtained with cyclosomes from M-phase cells (data not shown). Furthermore, incubation of cyclosomes from S-phase cells with protein kinase Cdk1/cyclin B markedly increased their stimulation by Cdc20 (Fig. 2b, compare lanes 2 and 6), and to a lesser extent by Cdh1 (Fig. 2b, lanes 3 and 7). In this experiment, it was important to terminate protein kinase action by staurosporine prior to the supplementation of Cdc20 or Cdh1, to prevent the inhibitory phosphorylations of these proteins (see below). Figure 2c shows that the above-described treatments indeed affected the phosphorylation state of the cyclosome, as indicated by the increased electrophoretic migration of cyclosome sub-

unit Cdc27 following phosphatase treatment of M-phase cyclosomes (Fig. 2c, lanes 1 and 2), and the retarded electrophoretic mobility of Cdc27 following incubation of S-phase cyclosomes with protein kinase Cdk1/cyclin B (Fig. 2c, lanes 3 and 4).

Phosphorylation of Cdc20 by Cdk1/cyclin B abrogates its stimulatory action and binding to cyclosome. We have next examined the effects of the phosphorylation of Cdh1 and Cdc20 on their action. It is well established that Cdh1 is expressed throughout the cell cycle and its activity is negatively regulated by phosphorylation (15–17). By contrast, Cdc20 is thought to be regulated primarily by its abundance: levels of Cdc20 are negligible in G1, rise to a peak in mitosis and fall rapidly afterwards (18, 25). Cell cycle-regulated phosphorylation of Cdc20 has also been described (10, 18, 19), but its role remained unclear. Kotani *et al.* (26) have reported that the phosphorylation by Cdk1/cyclin B of bacterially expressed, renatured Cdc20 converts it to a form that activates the cyclosome/APC. We have not been able to renature bacterially expressed Cdc20. Notably, it has been reported that in the mammalian cell cycle, the peak of the phosphorylation of Cdc20 actually preceded that of its abundance: Cdc20 was maximally phosphorylated in G2 and phosphorylation of Cdc20 dropped in the G2 to M-phase transition, while the levels of the Cdc20 protein were still rising (18). Other conditions under which Cdc20 was observed to be phosphorylated are associated with mitotic arrest. Thus, Cdc20/Fizzy is strongly phosphorylated in cells arrested in mitosis by the microtubule depolymerizing agent nocodazole (10), or in *Xenopus*

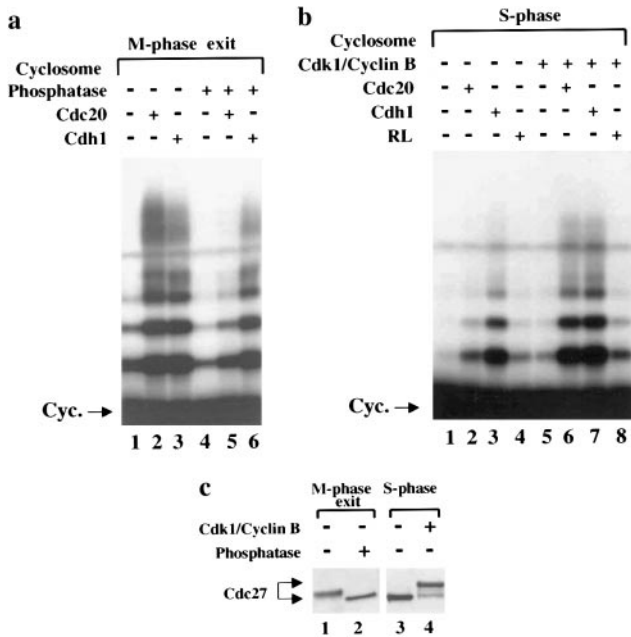


FIG. 2. Phosphorylation of mammalian cyclosome is required for its stimulation by Cdc20. (a) Cyclosomes from M-phase exit cells were first incubated (30°C, 30 min) in the presence (lanes 4–6) or absence (lanes 1–3) of 10 units lambda phosphatase (New England Biolabs) and 0.1 mM MnCl₂. Phosphatase action was terminated with 2 mM sodium vanadate, and subsequently Cdc20 or Cdh1 were added and cyclin-ubiquitin ligation assay was carried out. (b) Cyclosomes from S-phase cells were first incubated (30°C, 30 min) in the presence (lanes 5–8) or absence (lanes 1–4) of Cdk1/cyclin B (500 units). Protein kinase action was terminated with staurosporine (10 μM) and subsequently Cdc20, Cdh1, or reticulocyte lysate (“RL”) was added and cyclin-ubiquitin ligation assay was carried out in a further incubation. (c) The effects of treatments of cyclosomes from M-phase exit or S-phase cells with lambda phosphatase or protein kinase Cdk1/cyclin B, respectively, were tested by immunoblotting with an antibody directed against Cdc27 (Transduction Laboratories). Lanes 1 and 2 and 3 and 4 are from two different blots.

eggs naturally arrested in metaphase II by cytostatic factor (CSF) (19). Since the activity of the cyclosome/APC is inhibited under these conditions, the question arose whether the phosphorylation of Cdc20/Fizzy negatively regulates its action. We have examined this problem in our *in vitro* system. As shown in Fig. 3a, both Cdh1 and Cdc20 are phosphorylated *in vitro* by protein kinase Cdk1/cyclin B as indicated by retarded electrophoretic migration, though retardation was more prominent in the case of the former protein. The effects of these phosphorylations on the activities of Cdh1 and Cdc20 were examined in a two-stage incubation. In the first incubation, Cdh1, Cdc20 or an equivalent amount of reticulocyte lysate were incubated with MgATP, in the presence or absence of protein kinase Cdk1/cyclin B. Subsequently, protein kinase action was stopped by the addition of staurosporine, cyclosome preparation from M-phase cells was supplemented along with ¹²⁵I-cyclin, and cyclin-ubiquitin ligation was estimated following a sec-

ond incubation. As expected, phosphorylation of Cdh1 by Cdk1/cyclin B markedly inhibited its activity (Fig. 3b, compare lanes 2 and 7). Phosphorylation of Cdc20 by protein kinase Cdk1/cyclin B also abrogated its action to stimulate cyclosome activity (Fig. 3b, compare lanes 3 and 8). The slight stimulation of cyclosome activity caused by an amount of reticulocyte lysate equal to that present in preparations of Cdc20 or Cdh1 was not affected by incubation with the protein kinase.

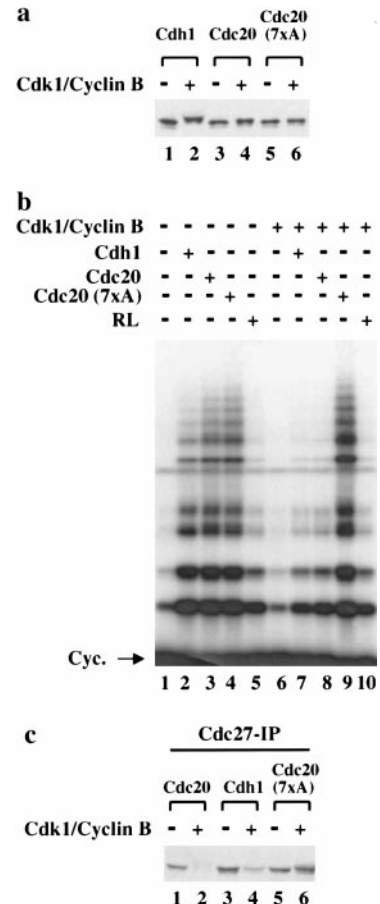


FIG. 3. Phosphorylation of Cdc20 by Cdk1/cyclin B abolishes its stimulatory action and binding to cyclosome. (a) ³⁵S-labeled proteins were incubated (30°C, 30 min) in the presence or absence of Cdk1/cyclin B (50 units/μl), as indicated, in a reaction mixture containing ATP-regenerating system and okadaic acid at concentrations similar to those used for the assay of cyclin-ubiquitin ligation (see Materials and Methods). (b) Preparations of Cdh1, Cdc20, Cdc20 (7xA) mutant, or reticulocyte lysate (“RL”) (0.5 μl, each) were first incubated (30°C, 30 min) in the presence or absence of Cdk1/cyclin B (500 units) in a reaction mixture similar to that described for the assay of cyclin-ubiquitin ligation (see Materials and Methods), except that cyclosome and ¹²⁵I-cyclin were omitted. Subsequently, protein kinase action was terminated with staurosporine (10 μM) and then cyclosomes from M-phase cells and ¹²⁵I-cyclin were added and cyclin-ubiquitin ligation was carried out in a further incubation of 60 min (c). The indicated ³⁵S-labeled proteins were incubated with or without Cdk1/cyclin B as described in (a) and their binding to cyclosome was determined by immunoprecipitation with anti-Cdc27 antibody, as described under Materials and Methods.

Since our preparation of Cdc20 contained reticulocyte lysate, it was possible that Cdk1/cyclin B phosphorylated a protein derived from reticulocyte lysate, which in turn inhibited the action of Cdc20. However, a mutant of Cdc20 in which 7 out of 8 potential phosphorylation sites had been converted to alanines ["Cdc20 (7xA)"] stimulated cyclosome activity, and its action was not diminished by prior incubation with Cdk1/cyclin B (Fig. 3b, lanes 4 and 9). This finding indicated that the observed effect is not indirect, but is due to phosphorylation of Cdc20. We have furthermore examined the effect of the phosphorylation of Cdc20 on its binding to cyclosome. As shown in Fig. 3c, incubation of both Cdh1 and Cdc20 with Cdk1/cyclin B markedly reduced their binding to cyclosome, tested by immunoprecipitation with an antibody directed against the Cdc27 subunit of the cyclosome/APC. By contrast, the binding to cyclosome of the Cdc20 (7xA) mutant was not decreased by incubation with the protein kinase. The slight retardation in electrophoretic mobility of the Cdc20 (7xA) mutant following incubation with the protein kinase (Figs. 3a and 3c, lanes 6) may be due to phosphorylation at the single remaining phosphorylation site at T109). We conclude that phosphorylation of Cdc20 abolishes its binding to cyclosome and its stimulatory effect on cyclosome activity, in a way similar to the known inhibitory phosphorylation of Cdh1.

Phosphorylation of Cdc20 and mitotic checkpoint control. The above findings raised the question of the possible role of the inhibitory phosphorylation of Cdc20 in cell cycle regulation. In early mitosis or under conditions that interfere with the normal assembly of the mitotic spindle, the cyclosome is kept inactive by the spindle checkpoint mechanism (20–22). It has been shown that the checkpoint protein Mad2 interacts with Cdc20 and prevents its action to stimulate the cyclosome/APC (23–25). It seemed possible that the phosphorylation of Cdc20 that occurs in early mitosis or in checkpoint-arrested cells, provides an additional safeguard to prevent premature or inappropriate activation of the cyclosome/APC under these conditions. To examine this notion, we have compared the effects of increasing concentrations of wild-type Cdc20 and of its nonphosphorylatable mutant on cyclin-ubiquitin ligation in extracts from nocodazole-arrested cells. As shown in Fig. 4, the addition of wild-type Cdc20 slightly stimulated cyclin-ubiquitin ligation in extracts from checkpoint-arrested cells. Similar concentrations of the Cdc20 (7xA) mutant markedly stimulated cyclin-ubiquitin ligation in the same extract. A likely explanation is that the action of wild type, but not of the nonphosphorylatable mutant of Cdc20, is prevented by the high activity of Cdk1/cyclin B present in extracts from nocodazole-arrested cells.

Our suggestion that phosphorylation of Cdc20 has a role in mitotic checkpoint control is based on *in vitro*

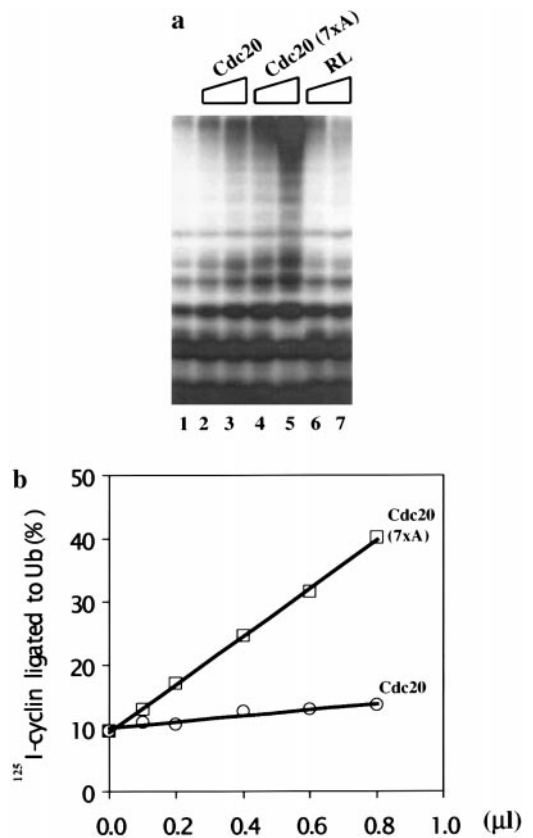


FIG. 4. Stimulation of cyclin-ubiquitin ligation in extracts from nocodazole-arrested cells by a nonphosphorylatable mutant of Cdc20. (a). All incubations contained extract (15 μg of protein) from nocodazole-arrested HeLa cells. The indicated preparations were supplemented in amounts of 0.3 μl (lanes 2, 4, 6) or 0.6 μl (lanes 3, 5, 7). Cyclin-ubiquitin ligation was determined as described under Materials and Methods. (b). Quantitation of a similar experiment.

experiments. However, we have shown that our *in vitro* system faithfully reproduces the *in vivo* selectivity of Cdc20 to activate the mitotic form of the cyclosome (Fig. 1). Furthermore, our findings are consistent with the observation that phosphorylation of Cdc20 precedes the peak of its accumulation in the mammalian cell cycle (18). The mechanism responsible for the dephosphorylation of Cdc20 in late mitosis or upon exit from mitotic checkpoint, remains to be elucidated. This mechanism may inhibit the phosphorylation of Cdc20 by the protein kinase, may stimulate its dephosphorylation, or may do both. It may resemble the network that activates the protein phosphatase Cdc14, a process responsible for the dephosphorylation and activation of Cdh1 following mitosis (27, 28).

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