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Direct activation of cdc2 with phosphatase: identification of p13^{suc1}-sensitive and insensitive steps

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In *Xenopus* oocytes, activation of MPF during prophase-metaphase transition is associated with the tyrosine dephosphorylation of the cdc2 protein. In vivo and in cell-free extracts kinase activation can be inhibited by excess p13^{suc1}, a subunit of the protein kinase. Here we have demonstrated that affinity-purified cdc2 from *Xenopus* prophase oocytes may be activated in vitro by exposure to potato acid phosphatase. In vitro, excess p13 does not inhibit tyrosine dephosphorylation of prophase cdc2, but nonetheless binds and prevents the activation of the enzyme. By contrast, fully activated enzyme from metaphase *Xenopus* eggs is insensitive to excess p13. These observations define a p13-sensitive state in the activation of fully active cdc2 that follows tyrosine dephosphorylation.

cdc2; p13^{suc1}; *Xenopus laevis* oocyte; Meiotic maturation; Tyrosine dephosphorylation

1. INTRODUCTION

In response to a variety of mitogenic agents the fully grown *Xenopus* oocyte enters M phase and matures into a metaphase-arrested egg. During the G2/M transition, an activity known as M-phase promoting factor (MPF) develops in the oocyte and is directly responsible for inducing the initiation of meiosis [1-4]. Two proteins have been shown to be components of MPF, the cdc2 protein kinase and mitotic cyclins [5-9]. Although a complex between cdc2 and cyclins forms progressively during interphase [9,10] it remains catalytically inactive until the initiation of mitosis. Activation has been associated with dephosphorylation of cdc2 on tyrosine and threonine residues [11-13], and in fission yeast a tyrosine phosphorylation site mutant initiates mitosis prematurely [14]. A further subunit of the cdc2 protein kinase is a 13 kDa protein (p13) encoded by the *suc1*$^+$ gene [15]. Although p13 appears to be an essential subunit of the protein kinase, excess p13 inhibits activation of MPF both in vivo and in complex cell-free lysates, but does not inhibit the function of the fully active M-phase enzyme [6,12]. p13-dependent inhibition of kinase activation is associated with failure of cdc2 tyrosine dephosphorylation in cell free system [12]. This led to the hypothesis that p13 might act either directly or indirectly to inhibit the cdc2 tyrosine phosphatase activity. Here we show that p13 inhibits cdc2 activation after the tyrosine dephosphorylation step.

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2. MATERIALS AND METHODS

2.1. *Oocyte preparation*

Xenopus laevis prophase oocytes were prepared as described [16] and were induced to mature by 1 μM progesterone. The soluble protein fractions were prepared in MPF extraction buffer [17] EB; 80 mM β-glycerophosphate pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$ supplemented with protease inhibitors (25 μg leupeptin/ml, 25 μg aprotinin/ml, 1 mM benzamidine, 10 μg pepstatin/ml, 10 μg soybean trypsin inhibitor/ml, 0.5 mM PMSF) as described [18], by centrifugation at 4°C for 90 min at 165 000 × *g*.

2.2. *Preparation and use of p13-Sepharose beads*

p13 was purified and conjugated to Sepharose as described previously [15]. After preincubation with Sepharose Cl-6B and centrifugation to remove nonspecific binding, the oocyte extracts (2 mg of proteins in 1 ml EB) were incubated overnight under constant rotation at 4°C with 20 μl of p13 Sepharose. After 3 min centrifugation at 1000 rpm the p13-Sepharose pellets were washed twice with 1 ml of EB, then either resuspended in 80 μl of Laemmli sample buffer [19] and boiled for 3 min or immediately used for phosphatase treatment or H1 kinase assay. Electrophoresis and Western blot were performed as described [10].

2.3. *Histone-H1 kinase assay*

p13-Sepharose pellets were washed 3 times in 1 ml of kinase buffer (50 mM TRIS (pH = 7.4), 10 mM $MgCl_2$, 1 mM DTT, 20 mM *p*-nitrophenylphosphate, 20 mM Naβ-glycerophosphate, 0.1 mM *o*-vanadate, and protease inhibitors as in EB and resuspended in 50 μl of kinase buffer containing 25 nM of a synthetic peptide mimicking the inhibitory site of cAMP-dependent protein kinase inhibitor [20], 0.5 mg H1 histone/ml (Boehringer), 0.1 mM cold ATP and 5 μCi of γ^{32}P-ATP (111 TBq/mmol; Dupont). After a 10 min incubation at 30°C, the reaction was stopped by adding 30 μl of Laemmli sample buffer and boiling for 3 min. After electrophoresis, the bands of H1 histone were excised from the gel and counted in 4 ml of Aquasol-2 (Dupont) in a LS 7000 Beckman counter.

2.4. *Phosphatase treatments*

p13-Sepharose precipitates were washed 3 times in acid phosphatase buffer (50 mM PIPES pH = 6.0, 0.1% β-mercaptoethanol) or

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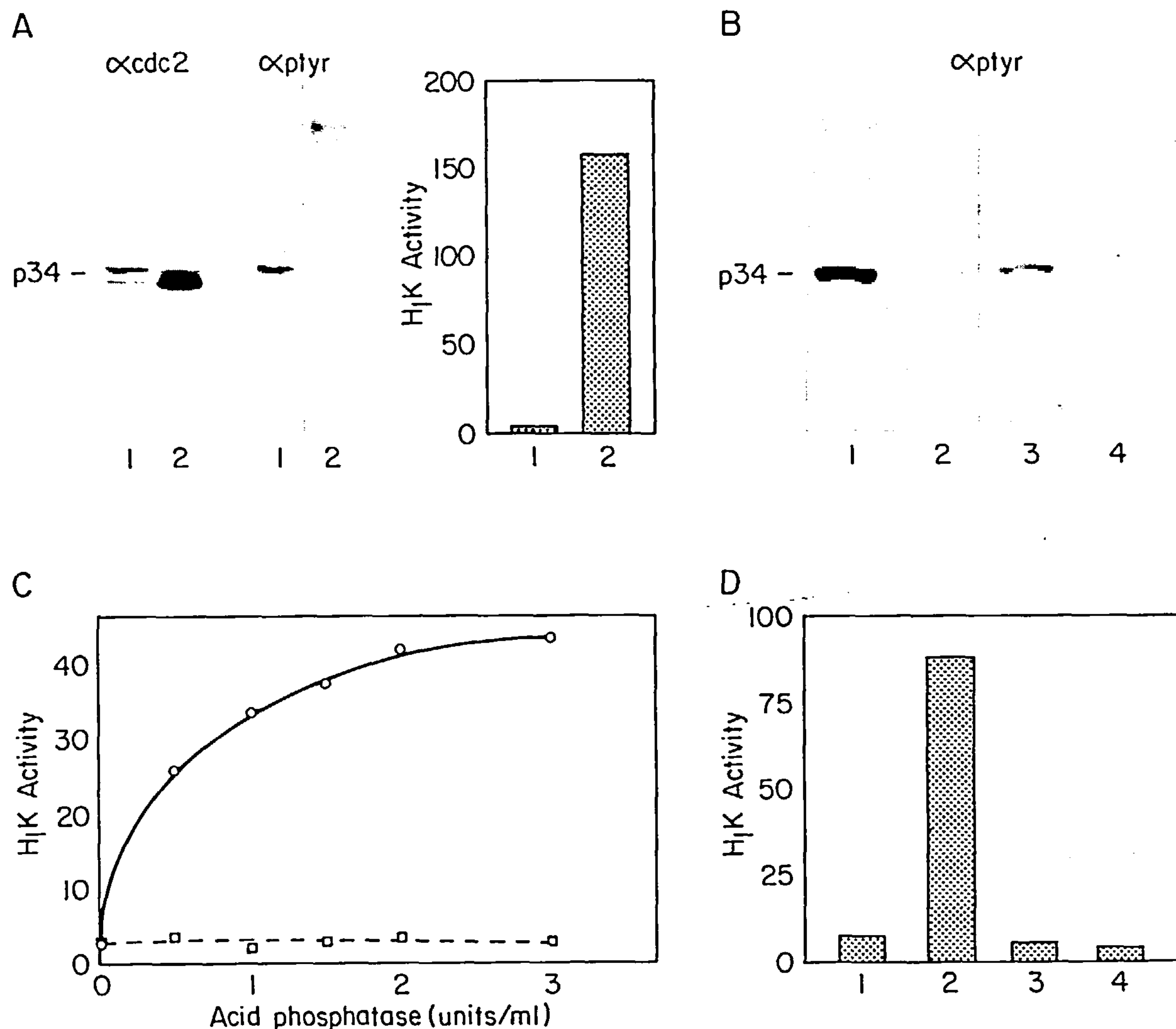


Fig.1. Activation of cdc2 with PAP. (A) Extracts of either prophase (lane 1) or metaphase (lane 2) oocytes were p13-Sepharose precipitated and immunoblotted with anti-cdc2 antibody (left panel) or anti-phosphotyrosine antibody (middle panel), or assayed for histone-H1 kinase activity (right panel) (in pmol phosphate incorporated in 5 μg H1 histone per 10 min incubation). (B) Anti-pTyr immunoblot of p13-Sepharose precipitate of oocyte extract, before or after exposure to PAP (lanes 1, 2) or alkaline phosphatase (lanes 3, 4). (C) Extract of prophase oocyte was loaded onto p13-Sepharose and histone-H1 kinase activity was assayed after exposure to increasing amounts of PAP, in the absence (line) or presence (dashed line) of phosphatase inhibitors. (D) p13-Sepharose precipitate of prophase oocytes assayed as histone kinase either directly or after exposure to PAP (lanes 1, 2) or before and after exposure to alkaline phosphatase (lanes 3, 4).

in alkaline phosphatase buffer (50 mM TRIS pH = 8.5, 1 mM DTT) both containing protease inhibitors as in EB. Precipitates were then further incubated for 15 min at 30°C in 40 μl of acid phosphatase buffer or alkaline phosphatase buffer, either in the absence or in the presence of potato acid phosphatase (Boehringer, Grade II) or alkaline phosphatase (from calf intestinal mucosa, type XXX-A; Sigma). After 4 washes in the kinase buffer, the p13-Sepharose precipitates were immunoblotted or assayed for the H1 kinase activity.

3. RESULTS AND DISCUSSION

3.1. *In vitro activation of cdc2 by phosphatase treatment*

p13 from fission yeast binds to *Xenopus* cdc2 [6,12] and p13-Sepharose can be used to deplete a cell extract of cdc2 and its associated proteins. By immunoblotting with anti-cdc2 antibody, a 34 kDa band was detected in the p13-Sepharose precipitate from either prophase oocyte or metaphase egg (Fig. 1A, left panel). As described previously [12], probing a similar immunoblot with anti-phosphotyrosine serum, revealed tyrosine phosphorylation of cdc2 in the oocyte but not the egg (Fig. 1A, center panel). The histone-H1 kinase activity was 56-fold higher in metaphase extracts than in prophase (Fig. 1A, right panel). It has been shown recently that p13-bound cdc2/cyclin from starfish oocytes can be partially activated by exposure to potato acid phosphatase (PAP) [21]. p13-precipitated extracts from *Xenopus* oocytes were exposed for 15 min to varying amounts of PAP (0.5–3.0 units/ml), washed in the presence of phosphatase inhibitors, and assayed for

histone-H1 kinase activity. PAP stimulated the enzyme 17-fold at the highest concentration used, but no activation was observed in the presence of phosphatase inhibitors (Fig. 1C). Exposure to the phosphatase resulted in tyrosine dephosphorylation (85–100% complete, Fig. 1B, lanes 1, 2) but no alteration in the level of cdc2 bound to p13-Sepharose (not shown). Although cdc2 was dephosphorylated, at least on tyrosine residues, the activated oocyte enzyme was not as potent as that from metaphase eggs. It has been demonstrated that specific removal of phosphotyrosine is unable to activate cdc2/cyclin from 3T3 fibroblasts [13], suggesting that tyrosine dephosphorylation is insufficient to activate the enzyme. To explore this in *Xenopus*, we exposed oocyte cdc2 to alkaline phosphatase, which can act as a phosphotyrosine phosphatase [22]. Although this phosphatase removed the tyrosine phosphate from cdc2 as efficiently as PAP (Fig. 1B, lanes 3, 4), it totally failed to activate the enzyme (Fig. 1D). This observation confirms that tyrosine dephosphorylation is insufficient to trigger the activation of the cdc2 kinase and

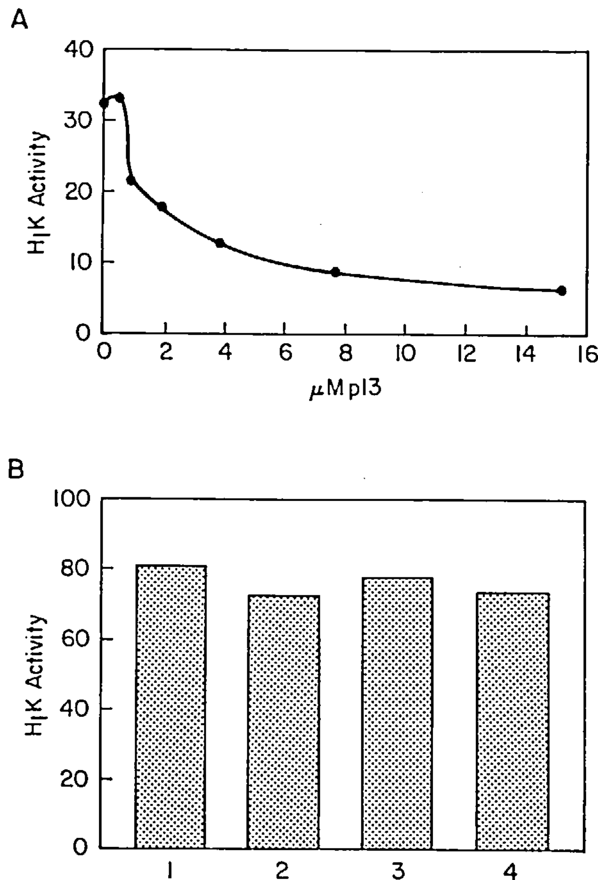
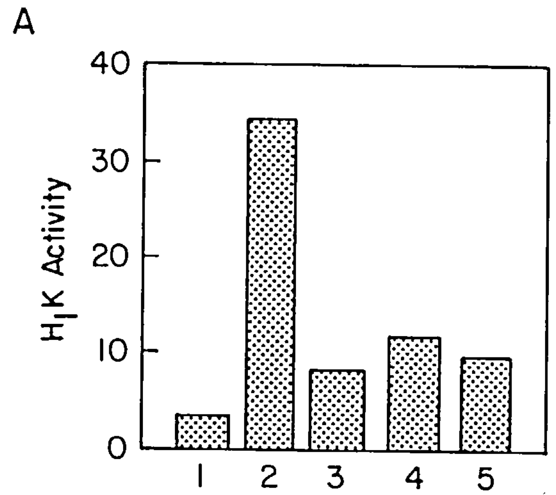


Fig.2. p13 inhibits activation of cdc2. (A) Histone-H1 kinase activity of prophase extract p13-Sepharose precipitated and exposed to 3 units/ml PAP in the presence of indicated amounts of soluble p13. (B) Histone-H1 kinase activity in metaphase extracts p13-Sepharose precipitated and exposed to no PAP (lane 1), 3 units/ml PAP (lane 2), 7.7 μM p13 (lane 3) or 3 units/ml PAP followed by 7.7 μM p13 (lane 4).



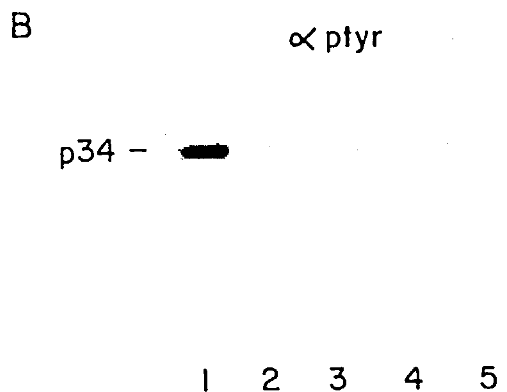


Fig.3. p13 does not inhibit tyrosine dephosphorylation of cdc2. Prophase extracts were precipitated with p13-Sepharose, washed in PAP buffer precipitates and treated as follows. No agents added (lane 1), 3 units/ml PAP (lane 2), 3 units/ml PAP and in the presence of 7.7 μM p13 (lane 3). Incubation with 7.7 μM p13, followed by extensive washing and then exposure to 3 units/ml PAP (lane 4). Exposure to 3 units/ml PAP, and after further washes, p13 was added at 7.7 μM (lane 5). Each reaction mix was then assayed for histone kinase (A) or subjected to immunoblotting with anti-phosphotyrosine antibody (B). (C) Anti-cdc2 immunoblot of oocyte lysate (lane 1), p13 precipitate of oocyte lysate (lane 2) or p13 precipitate of oocyte lysate after exposure to 7.7 μM p13 and subsequent washes with buffer (lane 3). This level of p13 fails to elute the bead-bound cdc2.

that other residues, probably threonine, are dephosphorylated by PAP as occurs in vivo. Conversely, we showed that histone-H1 kinase activity of cdc2 was strongly decreased in activated eggs in the presence of cycloheximide, in the absence of tyrosine rephosphorylation (not shown). Tyrosine phosphorylation is then not necessary for the in vivo inactivation of the kinase activity of cdc2 during the metaphase-interphase transition.

3.2. *p13 blocks the kinase activation of cdc2*

Although in each of the preceding experiments cdc2 has been bound to p13-Sepharose we further tested whether excess soluble p13 might inhibit kinase activation, mimicking the inhibition seen in whole cell mitotic extracts [12]. p13-Sepharose precipitates from oocytes were exposed to PAP in the presence of increasing concentrations of p13 (0–15 μM) and after washes the histone-H1 kinase activity was assayed. Soluble p13 inhibited activation of the kinase in a dose-dependent manner, up to a level of 80% (Fig. 2A). Immunoblotting showed that at the levels of soluble p13 used, cdc2 was not eluted from the p13-Sepharose (see Fig. 3C). Moreover, the fully activated H1 kinase from metaphase eggs is totally unaffected by the addition of p13, either in the presence or absence of PAP (Fig. 2B).

3.3. *p13 acts by binding to a second site*

To further explore the inhibitory action of p13, a prophase extract was p13-Sepharose precipitated, washed, and incubated with an excess of free p13. The pellet was then extensively washed in order to remove all the excess of free p13, and treated with PAP before assay of H1 kinase activity. Under these conditions, the phosphatase treatment failed to activate the kinase (Fig. 3A, lanes 1–4). We conclude from this experiment that p13 inhibits the in vitro activation of the kinase by stable binding to cdc2. Since the cdc2 protein is already bound to p13-Sepharose, the inhibitory p13 must bind cdc2 at a second site.

3.4. *p13 does not block cdc2 tyrosine dephosphorylation*

In an additional test of the inhibitory action of p13, we exposed p13-bound cdc2 to PAP, and after extensive washes, we added soluble p13. Unexpectedly, the kinase was inhibited (Fig. 3A, lane 5), even though tyrosine dephosphorylation could be shown to have occurred whether p13 was added before or after exposure to PAP (Fig. 3B). These observations demonstrate that excess soluble p13 inhibits activation of cdc2, not by preventing dephosphorylation but by binding to the protein. The present results do not contradict the observation that in cell-free extracts soluble p13 not only inhibits cdc2 activation but also tyrosine dephosphorylation [12]. Under these conditions, an autoactivation reaction occurs, during which small amounts of added

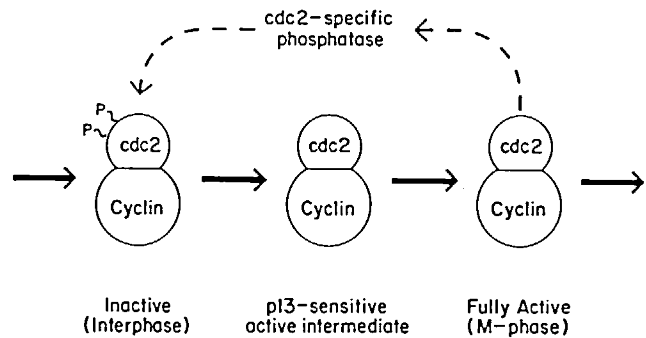


Fig.4. Model for activation of cdc2/cyclin. The cdc2/cyclin complex forms in interphase but is inactive. Activation occurs by tyrosine and threonine dephosphorylation. This step can be mimicked in vitro with PAP. In this state the enzyme is sensitive to p13. Full maturation of the enzyme in vivo involves further, as yet unidentified, steps that render it insensitive to p13. Active cdc2/cyclin is presumed to autoactivate the interphase enzyme further by activating a cdc2-specific phosphatase.

M-phase enzyme activate a much larger pool of interphase enzyme, presumably by stimulating a cdc2-specific phosphatase (Fig. 4). p13 appears to inhibit tyrosine dephosphorylation because it interferes with the autoactivation loop by inhibiting the activation of cdc2 after the dephosphorylation step. Thus, no further active enzyme is recruited and tyrosine dephosphorylation of the bulk of the cdc2 in the interphase extract fails to occur.

In summary, our results provide further direct biochemical evidence that dephosphorylation of the cdc2 protein is able to activate the kinase. However, dephosphorylation is followed by an additional step, necessary for full kinase activation and negatively regulated by excess p13. The molecular identity of this second step remains to be elucidated. It could be removal of phosphate by an endogenous phosphatase at sites other than those sensitive to PAP, or a quite different modification of the cdc2/cyclin complex might occur. The p13-mediated negative regulation of cdc2 activation appears to involve p13 binding to a second site on cdc2. The existence of two binding sites for p13 on the cdc2 protein could explain the complex and apparently contradictory data previously obtained, concerning the cellular function of p13. p13 might be an essential subunit of the protein kinase, for example being required for complex formation between cdc2 and cyclin, but binding of p13 at a secondary site could be inhibitory to enzyme activity. It remains to be established how many p13 subunits are present in the fully active M-phase enzyme in vivo.

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