Differential regulation of Cdc2 and Cdk2 by RINGO and cyclins

A. Karaiskou, L.H. Perez, I. Ferby, René Ozon, Catherine Jessus, A.R. Nebreda

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 38, Issue of September 21, pp. 36028–36034, 2001

Printed in U.S.A.

Differential Regulation of Cdc2 and Cdk2 by RINGO and Cyclins*[S]

Received for publication, May 23, 2001, and in revised form, July 12, 2001
Published, JBC Papers in Press, July 18, 2001, DOI 10.1074/jbc.M104722200

Anthi Karaiskou‡§, Laurent H. Perez‡, Ingvar Ferby‡, René Ozon§, Catherine Jessus§, and Angel R. Nebreda‡¶

From the ‡European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany and §Laboratoire de Physiologie de la Reproduction, Université Pierre et Marie Curie, ESA 7080, CNRS, Institut National de la Recherche Agronomique, 4 Place Jussieu, Boîte 13, 75005 Paris, France

Cyclin-dependent kinases (Cdks) are key regulators of the eukaryotic cell division cycle. Cdk1 (Cdc2) and Cdk2 should be bound to regulatory subunits named cyclins as well as phosphorylated on a conserved Thr located in the T-loop for full enzymatic activity. Cdc2- and Cdk2-cyclin complexes can be inactivated by phosphorylation on the catalytic cleft-located Thr-14 and Tyr-15 residues or by association with inhibitory subunits such as $p21^{Cip1}$. We have recently identified a novel Cdc2 regulator named RINGO that plays an important role in the meiotic cell cycle of *Xenopus* oocytes. RINGO can bind and activate Cdc2 but has no sequence homology to cyclins. Here we report that, in contrast with Cdc2-cyclin complexes, the phosphorylation of Thr-161 is not required for full activation of Cdc2 by RINGO. We also show that RINGO can directly stimulate the kinase activity of Cdk2 independently of Thr-160 phosphorylation. Moreover, RINGO-bound Cdc2 and Cdk2 are both less susceptible to inhibition by $p21^{Cip1}$, whereas the Thr-14/Tyr-15 kinase Myt1 can negatively regulate the activity of Cdc2-RINGO with reduced efficiency. Our results indicate that Cdk-RINGO complexes may be active under conditions in which cyclin-bound Cdks are inhibited and can therefore play different regulatory roles.

Cyclin-dependent kinases (Cdks)[1] are essential regulators of the eukaryotic cell division cycle. Cdks are catalytically inactive as monomers, and their enzymatic activity is modulated by association with other proteins as well as by both inhibitory and activating phosphorylations (1–4).

Activation of Cdks requires association with regulatory subunits named cyclins that are synthesized and degraded in a cell cycle-dependent manner (5, 6). Crystallographic studies have revealed that cyclin binding results in important structural modifications on the catalytic cleft of the Cdk molecule, allowing the correct folding for a better accessibility to ATP and substrates (7). In addition, cyclins have been shown to provide Cdks with targeting domains, responsible for substrate selection and thus biological specificity (8, 9). The Cdk-cyclin complex is fully active only after phosphorylation of a conserved Thr residue (Thr-161 in Cdc2 and Thr-160 in Cdk2) located on the T-loop, close to the catalytic cleft of the kinase (10–14). This phosphorylation is catalyzed by the Cdk-activating kinase (CAK), identified as a trimeric complex (Cdk7, cyclin H, and MAT1) in higher eukaryotes (15–19) or as a monomeric enzyme, named Cak1/Civ1, in budding yeast (20–22). CAKs appear to be constitutively active during the cell cycle (12, 17, 23).

The activity of Cdk-cyclin complexes can be inhibited by phosphorylation of residues present within the ATP-binding region of the Cdk, Thr-14 and Tyr-15 in higher eukaryotes (24, 25). Two kinases, Wee1 and Myt1, have been shown to phosphorylate Cdc2 either only on Thr-14 or Tyr-15 or on both residues, depending on the species (26–28). Both kinases are down-regulated by phosphorylation just before entry into the M phase of the cell cycle (28–30). Cdk-cyclin complexes can also associate with small inhibitory molecules, the CKIs (Cdk inhibitors) (31). CKIs interact both with the amino-terminal lobe of the Cdk and with the cyclin subunit (32), and they can inhibit cyclin-bound Cdks by disrupting the correct folding of the catalytic cleft of the kinase and by interfering with CAK-induced phosphorylation (reviewed in Ref.4).

During the progesterone-induced meiotic maturation (G_2/M phase progression) of *Xenopus* oocytes, two different Cdc2 populations need to be activated. In the G_2-arrested oocyte, about 10% of the Cdc2 is associated with cyclin B and phosphorylated on Thr-161 but maintained inactive by phosphorylation on Thr-14 and Tyr-15. The remaining 90% of the Cdc2 is free, not complexed with cyclins, but it also appears to be required for progesterone-triggered M phase entry. Thus, the G_2/M progression in oocytes involves dephosphorylation of Thr-14/Tyr-15 residues of the cyclin B-associated Cdc2, which is preceded by the activation of monomeric Cdc2 by newly synthesized proteins (reviewed in Ref. 33). This free Cdc2 recruited to newly formed complexes during maturation should be able to escape the mechanisms that normally ensure the inhibition of Cdc2-cyclin complexes in the G_2-arrested oocyte. A candidate for this triggering role is a recently identified protein named RINGO (rapid inducer of G_2/M progression in oocytes) (34) or Speedy (35). This protein is required for the meiotic G_2/M progression in *Xenopus* oocytes and can bind to and stimulate the kinase activity of Cdc2 (34) as well as bind to Cdk2 (35). Here we have shown that RINGO binding also activates Cdk2 and have analyzed the biochemical mechanisms that regulate Cdc2 and Cdk2 activation by RINGO. Our results indicate that RINGO-bound Cdc2 and Cdk2 are active in the absence of the conserved T-loop located Thr phosphorylation and at the same time are more resistant to negative regulation via inhibitory phosphorylations and association with CKIs.

* This work was supported by grants from the European Molecular Biology Laboratory (to A. N.) and CNRS, Université Pierre et Marie Curie, and by ARC Grant 5899 (to C. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at *http://www.jbc.org*) contains an illustration of the immunoblotting of different fractions of oocyte extracts using the indicated antibodies.

¶ To whom correspondence should be addressed. Tel.: 49-6221-387426; Fax: 49-6221-387166; E-mail: nebreda@EMBL-heidelberg.de.

[1] The abbreviations used are: Cdk, cyclin-dependent kinase; CKI, Cdk inhibitor; RINGO, rapid inducer of G_2/M progression in oocytes; GST, glutathione *S*-transferase; CAK, Cdk-activating kinase.

EXPERIMENTAL PROCEDURES

Bacterial Expression and Purification of Recombinant Proteins—MalE-RINGO was expressed in *Escherichia coli* and purified as described (34). The GST fusion proteins of *Xenopus* Cdc2 wild type, K33R, and T161A, *Xenopus* Cdk2 wild type, K33R, and T160A, human $p21^{Cip1}$, and human cyclin A were bacterially expressed and purified as described (36). Human His-cyclin B1 was expressed in baculovirus-infected insect cells and purified as described (37). Human Myt1 purified from baculovirus-infected insect cells was provided by R. Booher (38). Cak1/Civ1-His (both wild type and the kinase-dead mutant K31A) was purified from yeast and provided by C. Mann (20).

Microinjection and Fractionation of Xenopus Oocytes—Fully grown stage VI *Xenopus* oocytes were microinjected with 50 nl of the indicated recombinant proteins or *in vitro* transcribed capped mRNAs prepared using the MEGAscript kit (Ambion). The RINGO and Cdc2 constructs for *in vitro* transcription have been described previously (34, 39). The Cdc2 T161A mutant was prepared by polymerase chain reaction using as a template the same Cdc2 construct and the QuikChange site-directed mutagenesis kit (Stratagene). Oocyte lysates were prepared in 4 volumes of EB buffer (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.3, and protease inhibitors) and fractionated exactly as described in Ref.40 to obtain the 40–60% ammonium sulfate fraction F60, which was stored in aliquots at −80 °C.

Kinase Activation Assays in Oocyte Extracts (F60)—Aliquots of the F60 cytosolic fraction corresponding to 80 oocytes were thawed, and the ammonium sulfate pellet was dissolved in 400 μl of EB buffer. The reconstituted F60 was then concentrated on a Microcon-10 filter device (Amicon-Millipore) to a final volume of 80 μl, supplemented with 10 μl of 1 mM ATP and 10 μl of 0.5 mM protein kinase A inhibitor peptide (Sigma). In a typical reaction, 15 μl of freshly reconstituted F60 were preincubated with 10 μl of purified Cak1/Civ1 or $p21^{Cip1}$ for 15 min at 30 °C followed by a further incubation with 10 μl of His-cyclin B1 or MalE-RINGO for 15 min at 30 °C. Myt1 (10 μl) was added after cyclin B1 or RINGO. In some cases, Myt1 was added in the F60 in the presence of RINGO or cyclin B1 and 100 μM ATP. When any of these components were not included in the reaction, 10 μl of EB buffer were added. The histone H1 kinase activity was measured in a final volume of 50 μl in the presence of 100 μM ATP, 3 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham Pharmacia Biotech), 10 mM $MgCl_2$, and 0.3 mg/ml of histone H1 (Sigma). After 15 min at 30 °C, the kinase reactions were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. For quantification, the histone H1 bands were excised from the gel, and radioactive phosphate incorporation was determined by liquid scintillation counting (Wallac 1409 counter). In some experiments, phosphorylated bands were directly quantified on the gels using a Fuji phosphorimaging device.

Kinase Activation of Recombinant Cdc2 and Cdk2—Bacterially expressed GST·Cdc2 proteins could not directly be activated *in vitro* but require prior incubation in concentrated *Xenopus* oocyte extracts (34), perhaps for the correct folding of the proteins. The GST·Cdc2 proteins were then recovered on glutathione-Sepharose beads (10 μl, Amersham Pharmacia Biotech) and washed three times with EB buffer before being used for kinase reactions. In contrast, purified bacterially produced GST·Cdk2 was directly used for the Cdk2 activation assays. Typically 1–2 μM GST-fused Cdc2 or Cdk2 were used per assay following the same protocol as indicated above for the F60 cytosolic fraction except that we used a final volume of 12 μl.

Immunoprecipitation, Immunoblotting, and Glutathione-Sepharose Pull-down—Immunoprecipitations and immunoblottings were performed as described in Ref. 36, using the anti-Cdc2 monoclonal antibody A17 coupled to beads (Santa Cruz Biotechnology, Inc.), the anti-Cdc2 monoclonal antibody 3E1 (a kind gift from Julian Gannon and Tim Hunt), and affinity-purified rabbit anti-RINGO antibodies (34).

To recover GST·$p21^{Cip1}$ and its associated proteins, 45 μl of the F60 fraction were diluted to 100 μl with EB buffer, and then 10 μl of glutathione-Sepharose beads were added. After a 45-min incubation, the beads were recovered by centrifugation and washed three times. Both supernatant and bead fractions were analyzed by immunoblotting using the anti-Cdc2 antibody 3E1.

RESULTS

RINGO-induced Cdc2 Activation Is Independent of Thr-161 Phosphorylation—We have previously shown that purified recombinant RINGO can stimulate the kinase activity of Cdc2 expressed in insect cells or in *Xenopus* oocytes as well as bacterially produced GST·Cdc2 (34). As full activation of Cdc2-cyclin complexes requires phosphorylation of Cdc2 on Thr-161 (3, 4), we investigated whether this phosphorylation was also required for activation of Cdc2 by RINGO. We incubated purified RINGO or cyclin B1 proteins with the F60 fraction prepared from G_2-arrested oocyte extracts by ammonium sulfate precipitation, which contains only monomeric Cdc2 but not A- and B-type cyclins or Cdk2 (40) (see supplementary data). The experiment was performed in the absence or presence of the monomeric yeast Cdk-activating kinase Cak1/Civ1, which can efficiently phosphorylate Thr-161 in Cdc2 (20–22). When the F60 was preincubated in the presence of Cak1/Civ1, we observed a strong increase in the H1 kinase activity triggered by purified cyclin B1, as expected (Fig. 1, *A*, *B*, and *E*). The addition of RINGO to the F60 induced H1 kinase activity (Fig. 1, *C–E*). However, Cak1/Civ1 did not increase but rather partially inhibited the RINGO-induced H1 kinase activity in the same F60 fraction (Fig. 1, *C–E*). Cak1/Civ1 had the same

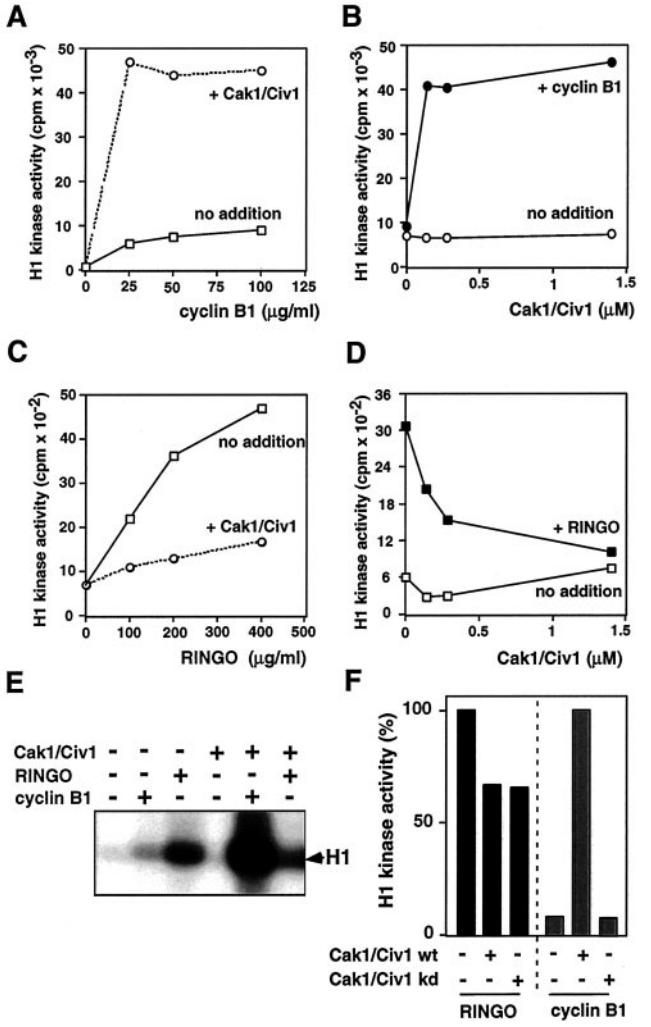

FIG. 1. **Effect of Cak1/Civ1 on the H1 kinase activity triggered by RINGO in free Cdc2-containing oocyte fractions.** *A–E*, oocyte cytosolic fraction F60 containing free Cdc2 preincubated with wild type (*wt*) Cak1/Civ1 at either 0.25 μM (*A*, *C*, and *E*) or the indicated concentrations (*B* and *D*) for 15 min at 30 °C in the presence of 100 μM ATP. MalE-RINGO or His-cyclin B1 was then added either at 2 μM (corresponding to 100 μg/ml and 125 μg/ml for MalE-RINGO and His-cyclin B1, respectively) (*B*, *D*, and *E*) or the indicated concentrations (*A* and *C*), and the samples were incubated for an additional 15 min at 30 °C prior to the histone H1 kinase assay. *F*, F60 fraction incubated with wild type or a kinase-dead (*kd*) Cak1/Civ1 mutant (0.25 μM), MalE-RINGO (2.5 μM), or His-cyclin B1 (1 μM), as indicated above. 100% histone H1 kinase activity corresponds to 1,500 and 10,800 cpm for RINGO and cyclin B1, respectively.

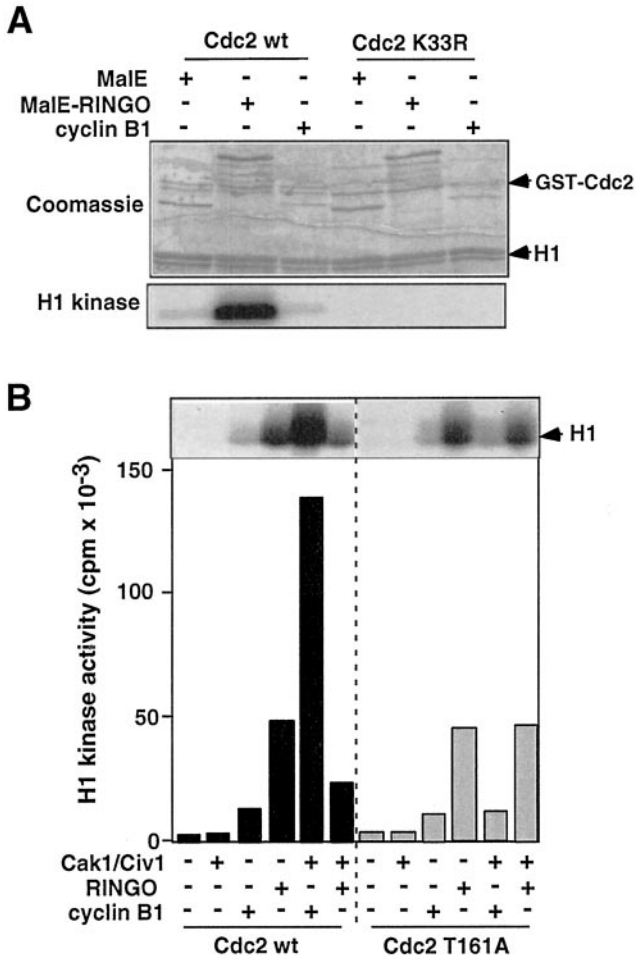

FIG. 2. **RINGO activates GST·Cdc2 independently of Thr-161 phosphorylation.** *A*, bacterially produced GST·Cdc2 wild type (*wt*) or K33R mutant (1 μM) preincubated in concentrated *Xenopus* oocyte extracts and recovered on glutathione-Sepharose beads. After washing, the beads were incubated in the presence of MalE, MalE-RINGO (2 μM), or His-cyclin B1 (1 μM) at 30 °C for 15 min prior to the histone H1 kinase assay. *B*, GST·Cdc2 wild type or T161A mutant (1 μM) processed as above and then incubated in the presence or absence of Cak1/Civ1 (0.25 μM) in a buffer containing 100 μM ATP for 15 min at 30 °C. MalE-RINGO or His-cyclin B1 (2 μM) was then added, and the samples were further incubated at 30 °C for 15 min prior to the histone H1 kinase assay.

effect when added to the extract before or after the addition of RINGO (data not shown). This suggests that T-loop phosphorylation is not needed for (and may even impair) Cdc2 activation by RINGO.

The reduction of RINGO-induced Cdc2 activity in the presence of Cak1/Civ1 may be attributable to an inhibitory effect of the phosphate bound to Thr-161 or to the interference between RINGO and Cak1/Civ1 for Cdc2 interaction. To distinguish between these two possibilities, we compared the effect of wild type Cak1/Civ1 and a kinase-inactive Cak1/Civ1 mutant on the RINGO-induced H1 kinase activity of the F60 fraction (Fig. 1*F*). Both wild type and kinase-dead Cak1/Civ1 proteins inhibited Cdc2 activity to the same extent, indicating that the inhibitory effect of Cak1/Civ1 on Cdc2-RINGO probably involves steric interference.

To confirm these results, we used bacterially produced GST·Cdc2 preincubated in concentrated *Xenopus* oocyte extracts (34). RINGO was able to stimulate the H1 kinase activity of wild type GST·Cdc2 but not of an inactive GST·Cdc2 K33R mutant protein processed in parallel (Fig. 2*A*). We then incubated wild type GST·Cdc2 or the GST·Cdc2 T161A mutant in the absence or presence of Cak1/Civ1 and assayed their kinase activity on histone H1 (Fig. 2*B*). As expected, cyclin B1 was able to activate the H1 kinase activity of GST·Cdc2, and this was strongly enhanced by the presence of Cak1/Civ1 in the case of Cdc2 wild type but not the Cdc2 T161A mutant (Fig. 2*B*). In contrast, RINGO activated both Cdc2 wild type and the T161A mutant to similar extents (Fig. 2*B*), and their kinase activities were not further increased in the presence of Cak1/Civ1. In fact, Cak1/Civ1 partially inhibited the RINGO-dependent activation of Cdc2 but did not inhibit the nonphosphorylable T161A mutant (Fig. 2*B*). This suggests that the inhibition might not require Cak1/Civ1 catalytic activity but is apparently abolished by replacing the Thr-161 of Cdc2 with an Ala residue. Altogether, the results indicate that the activation of Cdc2 by RINGO, either endogenous Cdc2 present in oocyte extracts or purified recombinant GST·Cdc2, can occur in the absence of Thr-161 phosphorylation. Furthermore, we reproducibly observed that Cdc2-RINGO complexes exhibit a lower specific activity toward histone H1 than Thr-161-phosphorylated Cdc2-cyclin B complexes.

Consistent with these *in vitro* results, we also found that overexpression in *Xenopus* oocytes of either wild type Cdc2 or the T161A Cdc2 mutant resulted in similar levels of RINGO-triggered H1 kinase activity (Fig. 3*A*). In contrast, the level of H1 kinase activity triggered by ectopic cyclin B1 was strongly reduced in Cdc2 T161A-expressing oocytes (Fig. 3*A*). These results indicate that RINGO is also able to induce Cdc2 activation independently of Thr-161-phosphorylation in oocytes. Moreover, RINGO binds Cdc2 under these conditions since we could co-immunoprecipitate Cdc2 and RINGO from oocyte extracts but only upon overexpression of the proteins (Fig. 3*B*).

RINGO-induced Cdk2 Activation Is Independent of Thr-160 Phosphorylation—RINGO has been shown to bind to Cdk2 upon overexpression in *Xenopus* oocytes, although it was not reported whether this resulted in the activation of Cdk2 by RINGO (35). We found that the kinase activity of bacterially produced GST·Cdk2 was directly activated (without any prior incubation in oocyte extracts) by recombinant RINGO, as determined by using histone H1 as an *in vitro* substrate (Fig. 4*A*). Moreover, the addition of Cak1/Civ1 did not modify the extent of RINGO-triggered Cdk2 activation, whereas Cak1/Civ1 was absolutely required for the cyclin A-induced activation of GST·Cdk2 (Fig. 4*A*). Under these conditions, we confirmed that Cak1/Civ1 phosphorylated Cdk2 with similar efficiency in the presence of either cyclin A or RINGO and that RINGO did not induce any Cdk2 autophosphorylation in the absence of Cak1/Civ1 (Fig. 4*B*). These results indicate that RINGO can directly activate Cdk2 and that this activation occurs in the absence of Thr-160 phosphorylation, as in the case of Cdc2. However, in contrast to Cdc2-cyclin B, the level of activation of Cdk2 was similar using either RINGO alone or cyclin A plus Cak1/Civ1. Interestingly, preincubation of Cdk2 with RINGO led to poor subsequent phosphorylation by Cak1/Civ1 in comparison with Cdk2 alone or Cdk2 preincubated with cyclin A (Fig. 4*C*, *upper panel*). This lower level of Cdk2 phosphorylation did not affect the H1 kinase activity of the Cdk2-RINGO complex (Fig. 4*C*, *lower panel*). These results indicate that RINGO binding may interfere with the accessibility of Cak1/Civ1 to Cdk2. To confirm that Cdk2 activation by RINGO is truly independent of Thr-160 phosphorylation, we used the Cdk2 T160A mutant. We found that RINGO stimulated the H1 kinase activity of the T160A mutant protein as efficiently as (or perhaps even slightly better than) in the case of the wild type Cdk2 (Fig. 4*D*). As a control, RINGO did not activate the Cdk2 K33R mutant.

Myt1 Inhibits Cdc2-RINGO with Reduced Efficiency—Because the phosphorylation of the conserved Thr located in the

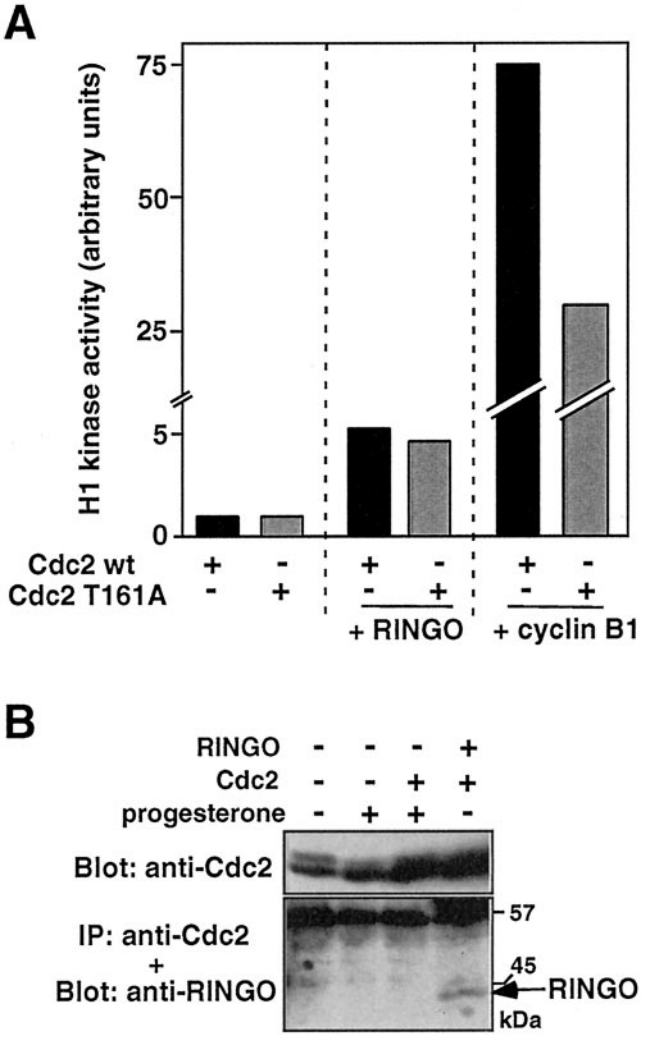

FIG. 3. **RINGO activates Cdc2 in *Xenopus* oocytes independently of Thr-161 phosphorylation.** *A*, oocytes injected with *in vitro* transcribed mRNAs encoding either Cdc2 wild type (*wt*) or Cdc2 T161A and incubated for 10 h prior to the injection of 50 ng of recombinant MalE-RINGO or His-cyclin B1. As a control, some oocytes were only injected with the Cdc2 mRNAs. After a further incubation for 2 h (the injected Cdc2 was overexpressed about 3-fold over the endogenous level), the oocyte lysates were prepared and assayed for histone H1 kinase activity, which was quantified using a phosphorimaging device. *B*, oocytes injected with *in vitro* transcribed Cdc2 mRNA and incubated overnight prior to the injection of RINGO mRNA or stimulation with progesterone (15 μM), as indicated. Lysates were prepared from 10 oocytes at the time of GVBD and immunoprecipitated with anti-Cdc2 antibodies followed by immunoblotting with anti-RINGO antibodies. Aliquots of the same lysates were also directly analyzed by immunoblotting using anti-Cdc2 antibodies.

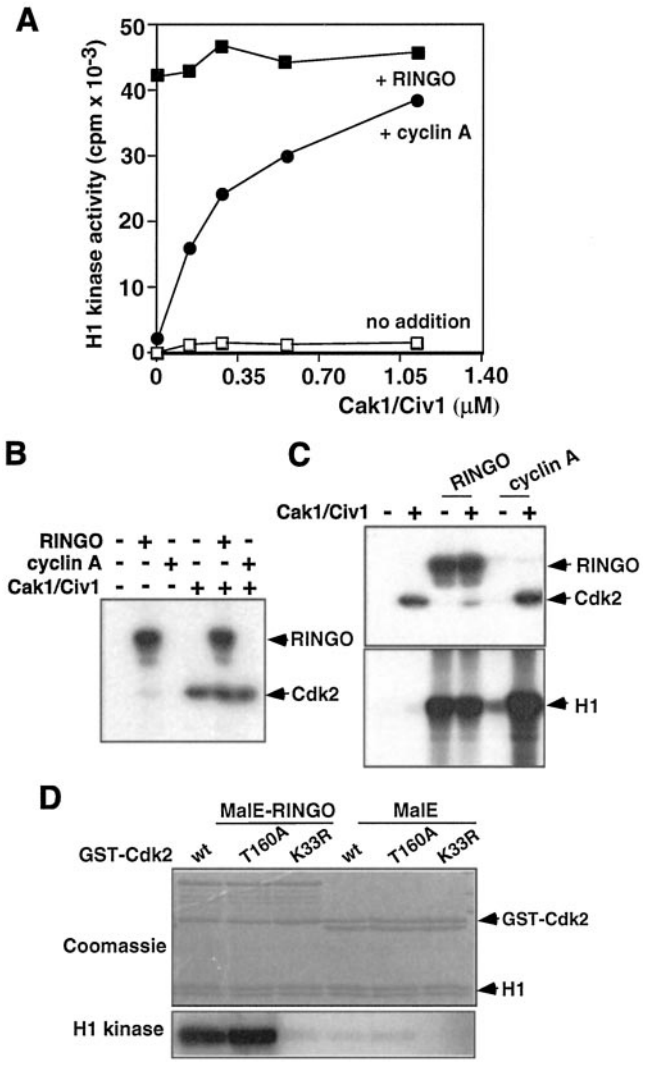

FIG. 4. **RINGO activates GST·Cdk2 independently of Thr-160 phosphorylation.** *A*, bacterially produced GST·Cdk2 (1 μM) incubated with either the indicated concentrations of Cak1/Civ1 or buffer for 15 min at 30 °C in the presence of 100 μM ATP. MalE-RINGO (2 μM) or GST·cyclin A (0.25 μM) was then added, and the samples were further incubated at 30 °C for 15 min prior to the histone H1 kinase assay. *B*, GST·Cdk2 (2 μM) incubated with MalE-RINGO (2.5 μM) or GST·cyclin A (0.5 μM) in the presence or absence of Cak1/Civ1 (0.25 μM) and with 100 μM ATP plus [γ-^{32}P]ATP for 15 min at 30 °C. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. *C*, GST·Cdk2 (2 μM) preincubated with MalE-RINGO (2 μM) or GST·cyclin A (0.5 μM) for 15 min at 30 °C and then Cak1/Civ1 (0.25 μM) added together with 100 μM ATP and [γ-^{32}P]ATP. The samples were further incubated at 30 °C for 15 min prior to analysis by SDS-polyacrylamide gel electrophoresis and autoradiography (*upper panel*). An identical set of samples was prepared in parallel and assayed for histone H1 kinase (*lower panel*). *D*, GST·Cdk2 wild type (*wt*) and the T160A and K33R mutants (1 μM) incubated in the presence of MalE or MalE-RINGO (2 μM) at 30 °C for 15 min prior to the histone H1 kinase assay.

T-loop was not required for the activation of Cdc2 and Cdk2 by RINGO, we investigated whether the inhibitory phosphorylation on Thr-14/Tyr-15 could affect the activity of the Cdc2-RINGO complex. We used the human Myt1 kinase, which has been shown to phosphorylate Cdc2-cyclin complexes preferentially on Thr-14 and to inhibit their kinase activity (26, 38). We found that the addition of 1 μM Myt1 to F60 oocyte extracts significantly reduced the H1 kinase activity triggered by either cyclin B1 or RINGO, suggesting that the Cdc2-RINGO complex is also subjected to inhibition by Myt1 (Fig. 5*A*). However, lower concentrations of Myt1 (below 0.3 μM) were more efficient inhibitors of histone H1 kinase activity of Cdc2-cyclin B than of Cdc2-RINGO. Using recombinant GST·Cdc2, we confirmed the reduced inhibitory effect of Myt1 on Cdc2-RINGO (Fig. 5*B*). The different concentrations of recombinant GST·Cdc2 (1 μM) *versus* endogenous Cdc2 present in the F60 (about 0.05 μM) could explain the differences in the degree of inhibition between both systems. We also found that the reduced inhibitory effect of Myt1 on Cdc2-RINGO correlated with less efficient phosphorylation of Cdc2 in the presence of RINGO than in the presence of cyclin B1 (data not shown).

p21^{Cip1} Is a Poor Inhibitor of Cdc2-RINGO and Cdk2-RINGO—Another important mechanism that down-regulates the activity of Cdks is their association with small proteins termed CKIs (4). We investigated the effect of the CKI family member p21^{Cip1} on the RINGO-induced activation of Cdc2. As

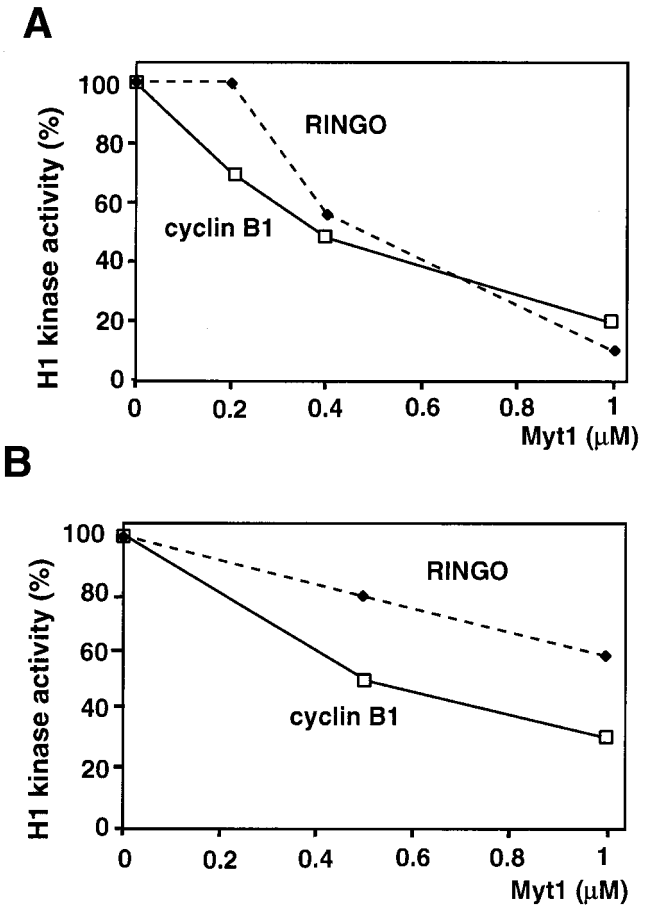

FIG. 5. **Myt1 inhibits the H1 kinase activity of Cdc2-RINGO with reduced efficiency.** The oocyte cytosolic fraction F60 containing free Cdc2 (*A*) or the GST·Cdc2 protein, preincubated in concentrated *Xenopus* oocyte extracts and recovered on glutathione-Sepharose beads (*B*), was incubated with MalE-RINGO or His-cyclin B1 (2 μM). The indicated concentrations of Myt1 were then added, and the samples were incubated for an additional 15 min at 30 °C in the presence of 200 μM ATP prior to the histone H1 kinase assay.

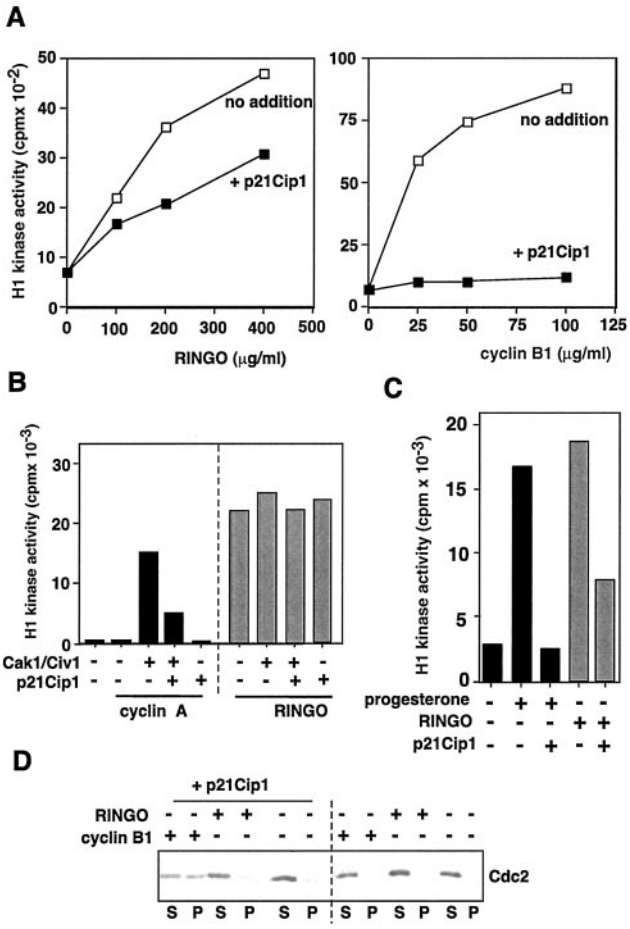

FIG. 6. **RINGO-bound Cdc2 and Cdk2 are less sensitive to p21^{Cip1} inhibition than the equivalent cyclin complexes.** *A*, oocyte cytosolic fraction F60 containing free Cdc2 preincubated with 1 μM GST·p21^{Cip1} for 15 min at 30 °C. Then, MalE-RINGO or His-cyclin B1 (2 μM) was added, and the samples were further incubated for 15 min at 30 °C followed by histone H1 kinase assay. *B*, bacterially produced GST·Cdk2 (1 μM) incubated with GST·p21^{Cip1} (1 μM) and/or Cak1/Civ1 (0.25 μM) for 15 min at 30 °C. MalE-RINGO (2 μM) or GST·cyclin A (0.25 μM) was then added, and the samples were further incubated at 30 °C for 15 min prior to the histone H1 kinase assay. *C*, oocytes injected with GST·p21^{Cip1} (1 μM) and 1 h later either incubated with progesterone or injected with MalE-RINGO (50 ng). Cdc2 activity was measured in cytosolic extracts 4 h later (corresponding to germinal vesicle breakdown time in uninjected, progesterone-treated oocytes) using histone H1 as an *in vitro* substrate. *D*, oocyte cytosolic fraction F60 containing free Cdc2 preincubated with GST·p21^{Cip1} (1 μM) for 15 min at 30 °C followed by the addition of MalE-RINGO or His-cyclin B1 (2 μM). Samples were incubated with glutathione-Sepharose beads, and the supernatant (*S*) and pellet (*P*) fractions were immunoblotted with an anti-Cdc2 antibody.

expected, when the F60 oocyte extract was preincubated with 1 μM p21^{Cip1} (a concentration sufficient to inhibit most of the known Cdks (41)), the cyclin B1-induced H1 kinase activation was completely blocked. However, the H1 kinase activity triggered by RINGO was only partially inhibited (Fig. 6*A*). This indicates that p21^{Cip1} has lower affinity and/or inhibitory efficiency for the Cdc2-RINGO complex than for the Cdc2-cyclin B complex. We also investigated the ability of p21^{Cip1} to inhibit the Cdk2-RINGO complex. We found that 1 μM p21^{Cip1} had no inhibitory effect on the GST·Cdk2-RINGO complex assembled *in vitro* using purified recombinant proteins, although the GST·Cdk2-cyclin A complex was efficiently inhibited by the same concentration of p21^{Cip1} (Fig. 6*B*). We also found evidence that Cdc2-RINGO complexes may be more resistant to p21^{Cip1} inhibition in *Xenopus* oocytes. Injection of p21^{Cip1} in oocytes prevented Cdc2 activation induced by progesterone. In contrast, RINGO was still able to induce significant Cdc2 activation in p21^{Cip1}-injected oocytes (Fig. 6*C*).

It therefore appears that both Cdc2- and Cdk2-RINGO complexes are less susceptible to inhibition by p21^{Cip1}. As the interaction mediated by the MRAIL motif of cyclins is essential for the ability of p21^{Cip1} to inhibit Cdks, a simple interpretation of our results is that RINGO lacks the MRAIL hydrophobic patch, leading to a weak Cdk-CKI association (32, 42). To test this possibility, GST·p21^{Cip1} was added to F60 (which contains only monomeric Cdc2) in the presence of either RINGO or cyclin B1 and then recovered on glutathione-Sepharose beads to investigate binding of Cdc2 to p21^{Cip1} by immunoblot (Fig. 6*D*). In these experiments, the addition of cyclin B1 led to the recovery of a significantly higher amount of Cdc2 associated with p21^{Cip1} than the addition of RINGO (Fig. 6*D*), suggesting that p21^{Cip1} has lower affinity for Cdc2-RINGO complexes.

DISCUSSION

Cdks are catalytically inactive as monomers and, for full activity, should be bound to a cyclin as well as phosphorylated on a Thr located on the T-loop, whereas the catalytic cleft-located Thr-14 and Tyr-15 residues should be dephosphorylated. We have investigated the mechanism of activation of Cdks by a new protein named RINGO that has no similarity to cyclins at the amino acid sequence level. We found that Cdk-

RINGO complexes respond to Cdk regulatory mechanisms in a different way than Cdk-cyclin complexes. Thus, the RINGO-induced kinase activity of Cdc2 and Cdk2 can bypass the requirement for phosphorylation of Thr located on the T-loop both *in vitro* and in oocytes. Moreover, Cdk-RINGO complexes appear to be less susceptible to inhibition by Thr-14/Tyr-15 phosphorylation and CKI binding.

How can the activity of Cdc2- and Cdk2-RINGO complexes be independent of Thr-160/161 phosphorylation, which is essential for full activation of these Cdks by cyclins? Crystallographic studies have revealed that phosphorylation of the Thr located in the T-loop induces subtle but important conformational changes; this phosphate acts as an organizing center, stabilizing the T-loop region (which probably participates in substrate recognition) as well as the Cdk-cyclin complex (14). In fact, one of the functions of the T loop phosphorylation may be to improve the binding of protein substrates independently of the catalytic activity *per se* (reviewed in Ref. 4). A hypothesis consistent with our results is that RINGO binding to Cdc2 and Cdk2 induces the conformational changes in the T-loop region that are normally induced upon Thr-160/161 phosphorylation. It should be noted that, in contrast to Cdc2 and Cdk2, other Cdks have been reported to be active in the absence of phosphorylation in the T-loop (for example the Cdk7-cyclin H-Mat1 complex (reviewed in Ref. 4)). This is also the case for Cdk5, which associates with and is activated by a brain-specific 35-kDa subunit, p35, as well as by its 25-kDa truncated form (p25) produced by the action of proteases (43, 44). Interestingly, a recent crystallographic study of the neuronal Cdk5-p25 complex reveals that the interaction between the p25 activator and the T-loop of Cdk5 results in a T-loop conformation closely resembling that of the Thr-160-phosphorylated Cdk2 associated with cyclin A.[2] These data suggest a common mechanism by which non-cyclin Cdk activators could bypass the requirement for T loop phosphorylation.

RINGO-bound Cdc2 and Cdk2 are also less susceptible to two mechanisms that negatively regulate the activity of Cdk-cyclin complexes: interaction with CKIs and phosphorylation on Thr-14/Tyr-15. This appears to correlate with weaker interaction with $p21^{Cip1}$ and less efficient phosphorylation by Myt1 of the Cdk-RINGO complexes. Interestingly, the similarity between RINGO and other non-cyclin Cdk activators also extends to the negative regulators as p25 allows Cdk5 to escape inhibition by the Tyr-15-specific kinase Wee1 (44) and by CKIs such as p27Kip1 (45). The absence of the MRAIL hydrophobic motif, typical of cyclins, could be responsible for the reduced inhibitory efficiency of $p21^{Cip1}$, as well as Myt1, on Cdc2-RINGO. It will be interesting to test the activity of Cdk-RINGO complexes on other substrates that require the MRAIL motif (for example the retinoblastoma family (42)). It is also conceivable that RINGO may provide Cdks with a substrate recognition and recruitment patch, perhaps also of a hydrophobic nature as in the MRAIL motif of cyclins (8).

Our biochemical findings confirm the properties expected for RINGO to have a triggering role in the activation of the meiotic cell cycle in *Xenopus* oocytes. Previous results indicated that newly synthesized RINGO protein is likely to be implicated in the activation of monomeric Cdc2 during the progesterone-induced oocyte maturation (34). About 90% of the Cdc2 present in the oocyte is not associated with cyclins (46), and only part of this monomeric Cdc2 appears to be already phosphorylated on Thr-161.[3] During the progesterone-triggered G_2/M transition, newly synthesized cyclin and RINGO proteins could target the two different Cdc2 populations, phosphorylated and nonphosphorylated on Thr-161, respectively. The Cdc2-RINGO complexes would be excellent triggers of the G_2/M progression of the oocyte. Firstly, they are poor substrates for Myt1, the kinase that phosphorylates Cdc2 on Thr-14/Tyr-15 and is probably responsible for the maintenance of Cdc2-cyclin B complexes inactive in G_2-arrested oocytes (36, 47). Secondly, Cdc2-RINGO complexes can also be active in the absence of Thr phosphorylation in the T-loop and can therefore bypass negative regulation by Thr-161 phosphatases potentially present in the oocyte, such as PP2C (48). Thus, the RINGO-activated Cdc2 generated in response to progesterone would not be affected by negative regulators of Cdc2 activity present in the oocyte and would be able to trigger the maturation process. Our results are also consistent with the possibility that newly synthesized RINGO may directly activate Cdk2, which is present in G_2-arrested oocytes and accumulates in response to progesterone (49). Previous work based on the use of the $p21^{Cip1}$ inhibitor had concluded that Cdk2 activity might be dispensable for the meiotic cell cycle in oocytes (50). However, these results need to be re-evaluated in light of our observations that $p21^{Cip1}$ is not an efficient inhibitor of Cdk2-RINGO. What the actual substrates of the RINGO-activated Cdc2 and Cdk2 could be in oocytes remains to be investigated. However, it should be noted that either the activation of Cdc25 or the inhibition of Myt1 should suffice to convert the inactive Cdc2-cyclin B complexes into active M-phase promoting factor and thus start the auto-amplification feedback loops that would induce entry into M-phase of meiosis of the oocyte.

Acknowledgments—We thank Robert Booher (Onyx, San Francisco) and Carl Mann (Service de Biochimie et de Génétique Moléculaire, Gif sur Yvette, France) for generously providing purified human Myt1 and yeast Cak1/Civ1 proteins, respectively. We also thank Andrea Musacchio (European Institute of Oncology, Milan, Italy) for communicating unpublished results, Véronique De Smedt and Robert Poulhe for helpful discussions, Giulio Superti-Furga and Gustavo Gutierrez for critically reading the manuscript, and Tim Hunt (Imperial Cancer Research Fund Clare Hall, South Mimms, UK), Christian Bréchot (Institut Necker/Pasteur, Paris, France), and David Beach (Institute of Child Health, London, UK) for providing GST fusion constructs.

REFERENCES

1. Lees, E. (1995) *Curr. Opin. Cell Biol.* **7,** 773–780
2. Pines, J. (1995) *Biochem. J.* **308,** 697–711
3. Johnson, L. N., Noble, M. E., and Owen, D. J. (1996) *Cell* **85,** 149–158
4. Morgan, D. O. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 261–291
5. Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D., and Hunt, T. (1983) *Cell* **33,** 389–396
6. Swenson, K. I., Farrell, K. M., and Ruderman, J. V. (1986) *Cell* **47,** 861–870
7. Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. (1995) *Nature* **376,** 313–320
8. Schulman, B. A., Lindstrom, D. L., and Harlow, E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10453–10458
9. Cross, F. R., Yuste-Rojas, M., Gray, S., and Jacobson, M. (1999) *Mol. Cell* **4,** 11–19
10. Booher, R., and Beach, D. (1986) *Mol. Cell. Biol.* **6,** 3523–3530
11. Gould, K. L., Moreno, S., Owen, D. J., Sazer, S., and Nurse, P. (1991) *EMBO J.* **10,** 3297–3309
12. Solomon, M. J., Lee, T., and Kirschner, M. W. (1992) *Mol. Biol. Cell* **3,** 13–27
13. Gu, Y., Rosenblatt, J., and Morgan, D. O. (1992) *EMBO J.* **11,** 3995–4005
14. Russo, A. A., Jeffrey, P. D., and Pavletich, N. P. (1996) *Nat. Struct. Biol.* **3,** 696–700
15. Fesquet, D., Labbé, J. C., Derancourt, J., Capony, J. P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Dorée, M., and Cavadore, J. C. (1993) *EMBO J.* **12,** 3111–3121
16. Poon, R. Y. C., Yamashita, K., Adamczewski, J. P., Hunt, T., and Shuttleworth, J. (1993) *EMBO J.* **12,** 3123–3132
17. Fisher, R. P., and Morgan, D. O. (1994) *Cell* **78,** 713–724
18. Makela, T. P., Tassan, J. P., Nigg, E. A., Frutiger, S., Hughes, G. J., and Weinberg, R. A. (1994) *Nature* **371,** 254–257
19. Devault, A., Martinez, A. M., Fesquet, D., Labbé, J. C., Morin, N., Tassan, J. P., Nigg, E. A., Cavadore, J. C., and Dorée, M. (1995) *EMBO J.* **14,** 5027–5036
20. Thuret, J. Y., Valay, J. G., Faye, G., and Mann, C. (1996) *Cell* **86,** 565–576
21. Kaldis, P., Sutton, A., and Solomon, M. (1996) *Cell* **86,** 553–564
22. Espinoza, F. H., Farrell, A., Erdjument-Bromage, H., Tempst, P., and Morgan, D. O. (1996) *Science* **273,** 1714–1717
23. Ross, K. E., Kaldis, P., and Solomon, M. J. (2000) *Mol. Biol. Cell* **11,** 1597–1609
24. Gould, K. L., and Nurse, P. (1989) *Nature* **342,** 39–45

[2] A. Musacchio, personal communication.

[3] V. De Smedt, R. Poulhe, X. Cayla, A. Karaiskou, C. Jessus, and R. Ozon, submitted for publication.

25. Krek, W., and Nigg, E. (1991) *EMBO J.* **10,** 305–316
26. Liu, F., Stanton, J. J., Wu, Z. Q., and Piwnica-Worms, H. (1997) *Mol. Cell. Biol.* **17,** 571–583
27. Atherton-Fessler, S., Liu, F., Gabrielli, B., Lee, M. S., Peng, C. Y., and Piwnica-Worms, H. (1994) *Mol. Biol. Cell* **5,** 989–1001
28. Mueller, P. R., Coleman, T. R., Kumagai, A., and Dunphy, W. G. (1995) *Science* **270,** 86–90
29. Mueller, P. R., Coleman, T. R., and Dunphy, W. G. (1995) *Mol. Biol. Cell* **6,** 119–134
30. Tang, Z. H., Coleman, T. R., and Dunphy, W. G. (1993) *EMBO J.* **12,** 3427–3436
31. Pines, J. (1994) *Trends Biochem. Sci.* **19,** 143–145
32. Chen, J., Saha, P., Kornbluth, S., Dynlacht, B. D., and Dutta, A. (1996) *Mol. Cell. Biol.* **16,** 4673–4682
33. Nebreda, A. R., and Ferby, I. (2000) *Curr. Opin. Cell Biol.* **12,** 666–675
34. Ferby, I., Blazquez, M., Palmer, A., Eritja, R., and Nebreda, A. R. (1999) *Genes Dev.* **13,** 2177–2189
35. Lenormand, J. L., Dellinger, R. W., Knudsen, K. E., Subramani, S., and Donoghue, D. J. (1999) *EMBO J.* **18,** 1869–1877
36. Palmer, A., Gavin, A. C., and Nebreda, A. R. (1998) *EMBO J.* **17,** 5037–5047
37. Kumagai, A., and Dunphy, W. G. (1997) *Methods Enzymol.* **283,** 564–571
38. Booher, R. N., Holman, P. S., and Fattaey, A. (1997) *J. Biol. Chem.* **272,** 22300–22306
39. Nebreda, A. R., Gannon, J. V., and Hunt, T. (1995) *EMBO J.* **14,** 5597–5607
40. Karaiskou, A., Jessus, C., Brassac, T., and Ozon, R. (1999) *J. Cell Sci.* **112,** 3747–3756
41. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993) *Nature* **366,** 701–704
42. Adams, P. D., Sellers, W. R., Sharma, S. K., Wu, A. D., Nalin, C. M., and Kaeling, W. G. (1996) *Mol. Cell. Biol.* **16,** 6623–6633
43. Lew, J., Huang, Q. Q., Qi, Z., Winkfein, R. J., Aebersold, R., Hunt, T., and Wang, J. H. (1994) *Nature* **371,** 423–426
44. Poon, R. Y., Lew, J., and Hunter, T. (1997) *J. Biol. Chem.* **272,** 5703–5708
45. Lee, M. H., Nikolic, M., Baptista, C. A., Lai, E., Tsai, L. H., and Massague, J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 3259–3263
46. Kobayashi, H., Minshull, J., Ford, C., Golsteyn, R., Poon, R., and Hunt, T. (1991) *J. Cell Biol.* **114,** 755–765
47. Nakajo, N., Yoshitome, S., Iwashita, J., Iida, M., Uto, K., Ueno, S., Okamoto, K., and Sagata, N. (2000) *Genes Dev.* **14,** 328–338
48. Cheng, A., Ross, K. E., Kaldis, P., and Solomon, M. J. (1999) *Genes Dev.* **13,** 2946–2957
49. Rempel, R. E., Sleight, S., B, and Maller, J. L. (1995) *J. Biol. Chem.* **270,** 6843–6855
50. Furuno, N., Ogawa, Y., Iwashita, J., Nakajo, N., and Sagata, N. (1997) *EMBO J.* **16,** 3860–3865

Differential Regulation of Cdc2 and Cdk2 by RINGO and Cyclins

Anthi Karaiskou, Laurent H. Perez, Ingvar Ferby, René Ozon, Catherine Jessus and Angel R. Nebreda

J. Biol. Chem. 2001, 276:36028-36034.
doi: 10.1074/jbc.M104722200 originally published online July 18, 2001
