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Requirement for Phosphorylation of Cyclin B1 for *Xenopus* Oocyte Maturation

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Maturation-promoting factor, consisting of *cdc2* protein kinase and a regulatory B-type cyclin, is a universal regulator of meiosis and mitosis in eukaryotes. In *Xenopus*, there are two subtypes of B-type cyclins, designated B1 and B2, both of which are phosphorylated. In this study, we have investigated the biological significance of this phosphorylation for *Xenopus* cyclin B1 during meiotic maturation. We have used a combination of site-directed mutagenesis and phosphopeptide-mapping to identify serine residues 2, 94, 96, 101, and 113 as presumptive phosphorylation sites, and together these sites account for all cyclin B1 phosphorylation in oocytes before germinal vesicle breakdown (GVBD). Single Ser→Ala mutants as well as multiple site mutants have been constructed and characterized. Phosphorylation of cyclin B1 appears to be required for *Xenopus* oocyte maturation, based on the significantly diminished ability of the quintuple Ala mutant to induce oocyte maturation. Furthermore, partial phosphorylation of these five sites is sufficient to meet this requirement. Phosphorylation of cyclin B1 is not required for *cdc2* kinase activity, for binding to *cdc2* protein, for stability of cyclin B1 before GVBD, or for destruction of cyclin B1 after GVBD or after egg activation. A quintuple Glu mutant was also constructed, with serine residues 2, 94, 96, 101, and 113 mutated to Glu. In contrast to the quintuple Ala mutant, the quintuple Glu mutant was able to induce oocyte maturation efficiently, and with more rapid kinetics than wild-type cyclin B1. These data confirm that phosphorylation, as mimicked by Ser→Glu mutations, confers enhanced biological activity to cyclin B1. Possible roles of cyclin B1 phosphorylation are discussed that might account for the increased biological activity of the quintuple Glu mutant.

INTRODUCTION

All eukaryotic cells use a common mechanism to regulate the onset of meiosis and mitosis. The key component in this mechanism is maturation-promoting factor (MPF). MPF consists of two subunits, *cdc2* kinase and either cyclin B1 or B2, which form a 1:1 complex (Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Draetta *et al.*, 1989; Meijer *et al.*, 1989). MPF was originally characterized as an activity present in unfertilized *Xenopus* eggs that could induce germinal vesicle breakdown (GVBD) when microinjected into resting oocytes (Masui and Markert, 1971). Subsequent work by many groups has characterized a variety of *cdc2* and cyclin-related proteins (reviewed in Pines, 1993; Sherr, 1993). The *cdc2* protein has been shown to contain three regulatory phosphorylation sites. These are Thr14 and Tyr15, both of which play a negative regulatory role, and Thr161, which plays a positive regulatory role (Ducommun *et al.*, 1991; Gould *et al.*, 1991a; Krek and Nigg, 1991a,b; Norbury *et al.*, 1991; Pickham *et al.*, 1992; Solomon *et al.*, 1992). Tyr15 is phosphorylated by *wee1* kinase (Russell and Nurse, 1987; Parker *et al.*, 1992; Parker and Piwnica-Worms, 1992), and Thr161 is phosphorylated by *cdc2*-activating kinase (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993). Dephosphorylation of Tyr15 and possibly Thr14 is controlled by the *cdc25* phosphatase (Gautier *et al.*, 1991; Gould *et al.*, 1991b; Millar *et al.*, 1991; Lee *et al.*, 1992).

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B-type cyclins are positive regulators of *cdc2* kinase and function in meiosis and mitosis, in contrast to other cyclins that may function in G1 or at the G1-S transition (reviewed in Draetta, 1993; Pines, 1993; Sherr, 1993; Solomon, 1993). B-type cyclins accumulate in interphase, peak at metaphase, and are rapidly degraded through a ubiquitin-dependent pathway during the metaphase-anaphase transition (Evans *et al.*, 1983; Minshull *et al.*, 1989; Solomon *et al.*, 1990; Glotzer *et al.*, 1991). Destruction of cyclins is a crucial event in exiting metaphase and entering the next interphase (Murray *et al.*, 1989; Luca *et al.*, 1991; Hunt *et al.*, 1992; Holloway *et al.*, 1993).

Stage VI *Xenopus* oocytes represent the final stage of oogenesis and are ready to mature upon stimulation by the physiological hormone progesterone (Dumont, 1972). In stage VI *Xenopus* oocytes, B-type cyclins and *cdc2* exist in an inactive pre-MPF complex, although there is also a 10- to 100-fold excess of free *cdc2* protein compared with B-type cyclins (Reynhout and Smith, 1974; Drury and Schorderet-Slatkine, 1975; Gautier and Maller, 1991; Kobayashi *et al.*, 1991b). In *Xenopus*, there are two subtypes of B-type cyclins, designated B1 and B2 (Minshull *et al.* 1989). During progesterone-stimulated *Xenopus* oocyte maturation, there is increased synthesis of both cyclin B1 and B2, with cyclin B1 levels increasing 3- to 10-fold (Kobayashi *et al.*, 1991b). In starfish oocytes, a nuclear factor has been implicated in increasing specific translation of cyclin B (Galas *et al.*, 1993). This increased synthesis of cyclin B may control the correct timing of the first meiotic cleavage. In goldfish oocytes, synthesis of new cyclin B is required for oocyte maturation (Katsu *et al.*, 1993). Microinjection of cyclin B1, B2, or even cyclin A into arrested *Xenopus* oocytes induces oocyte maturation (Swenson *et al.*, 1986; Westendorf *et al.*, 1989; Freeman *et al.*, 1991). During progesterone-stimulated oocyte maturation, both cyclin B1 and B2 are partially degraded shortly after GVBD (Kobayashi *et al.*, 1991b). This degradation is accompanied by a drop of MPF kinase activity, signalling exit from the first meiotic metaphase. After egg fertilization, both cyclin B1 and B2 are completely and quickly destroyed, signifying completion of the second meiotic division (Murray *et al.*, 1989).

Previously, the appearance of histone H1 kinase activity in cleaving sea urchin eggs was shown to correlate with phosphorylation of the cyclin subunit of MPF (Meijer *et al.*, 1989). Furthermore, in both *Xenopus* oocytes and eggs, B-type cyclins have been reported to be phosphorylated (Gautier and Maller, 1991). Ser94 and/or Ser96 of *Xenopus* cyclin B1 were previously identified as phosphorylation sites in *Xenopus* egg extracts (Izumi and Maller, 1991). In addition, Ser90 of *Xenopus* cyclin B2 was identified as a phosphorylation site in both maturing oocyte extracts and egg extracts (Izumi and Maller, 1991; Kobayashi *et al.*, 1991a). However, no function for these phosphorylation sites has been determined.

In this investigation, we have studied the phosphorylation of *Xenopus* cyclin B1 and its biological functions during *Xenopus* oocyte maturation. We have identified serine residues 2, 94, 96, 101, and 113 as presumptive phosphorylation sites, and together these sites account for all detectable cyclin B1 phosphorylation in oocytes before GVBD. Surprisingly, using a quintuple Ala mutant with Ser→Ala mutations at residues 2, 94, 96, 101, and 113, our data suggest that phosphorylation of cyclin B1 does not affect *cdc2* kinase activity, *cdc2* binding to cyclin B1, stability of cyclin B1 in immature oocytes, or the normal degradation of cyclin B1. Nonetheless, the quintuple Ala mutant was very limited in its ability to induce oocyte maturation, suggesting a key role for phosphorylation in the biological activity of cyclin B1. When a quintuple Glu mutant was constructed, with serine residues 2, 94, 96, 101, and 113 mutated to Glu to mimic phosphoserine residues, this mutant was able to induce oocyte maturation efficiently, and with more rapid kinetics than wild-type cyclin B1. This is consistent with a significant biological role for cyclin B1 phosphorylation.

MATERIALS AND METHODS

Mutagenesis

Xenopus cyclin B1 cDNA was subcloned from pGEM-XLB1 (kindly provided by T. Hunt (ICRF Clare Hall Labs, United Kingdom); Minshull *et al.*, 1989) into pBluescript KS(−) (Stratagene, San Diego, CA) by using the restriction sites *Eco*RI and *Bam*HI. For construction of the single Ser→Ala mutants, single-stranded uracil-containing cyclin B1 DNA was isolated and mutagenized as described previously (Zoller and Smith, 1983; Kunkel, 1985). For example, to construct the S2A mutant, we used the following oligonucleotide: 5′-CGCTTGAGAA AATGGCGCTA CGAGTC, which encodes an Ala in place of Ser2. The sequences of the other 18 mutagenic oligonucleotides will not be given here; however, details are available upon request. Restriction fragments containing the mutated DNAs were sequenced and then subcloned as appropriate into pSB5-cyclinB1, where pSB5 is a derivative of pSP64(polyA) (Promega, Madison, WI) in which the linearization site *Eco*RI has been changed to *Xho*I.

Unless otherwise noted, all cyclin B1 derivatives utilized in this work contain a C-terminal epitope tag to allow specific recovery of the mutant cyclin B1 protein. As a result, the last three amino acids of cyclin B1 (PLM) were removed and replaced by the 15-mer epitope tag GLEVIVVPHSLPFML, which has been described previously in work from this laboratory (Pickham *et al.*, 1992). Some derivatives, such as those described in Figure 10C, were also constructed with a C-terminal epitope tag derived from the glycoprotein G of vesicular stomatitis virus, against which mAB P5D4 is available (Kreis and Lodish, 1986).

The triple mutant S2A/S101A/S113A was made by exchanging restriction fragments of the corresponding single mutants. However, the multiple site mutants S94A/S96A, S93A/S94A/S96A, and S2A/S94A/S96A/S101A/S113A required synthesis of a small restriction fragment encoding contiguous mutations. For example, to construct the S94A/S96A double mutant, the following complementary oligonucleotides were synthesized: 1) 5′-AGGCAAAACC TGTTGACAAA TTGTTGGAGC CTCTTAAAGT GATAGAAGAG AATGTTTGCC CTAAACCTGC TCAGGTTGAA CCCAGTGCAC

CAGCGC; and 2) 5'- TGGTGCACTG GGTTCAACCT GAGCAG-GTTT AGGGCAAACA TTCTCTTCTA TCACTTTAAG AGGCTC-CAAC AATTTGTCAA CAGGTTTTGC CTTCT. When annealed together, these oligonucleotides form a small restriction fragment with *Bgl*I and *Hae*II cohesive ends, coding for residues 63–96. For brevity, the sequences of the oligos used to construct the other multiple site mutants will not be presented here. However, like the S94A/S96A mutant, the synthetic restriction fragment encoding the S93A/S94A/S96A mutations also possessed *Bgl*I and *Hae*II cohesive ends, whereas the fragment encoding the S94A/S96A/S101A mutations possessed *Bgl*I and *Sst*II cohesive ends. These fragments were then ligated into the DNA encoding cyclin B1 (either wild type or else containing additional appropriate mutations) in pSB5-derived vectors. The quintuple Glu mutant was constructed by similar approaches using long synthetic oligonucleotides encoding the desired Ser→Glu mutations. Isolation of the correct insert was verified for each mutant by DNA sequencing.

Oocyte Microinjection

Adult female *Xenopus* were obtained from *Xenopus* I (Ann Arbor, MI). Ovaries were dissected 1 day before microinjections; 10–15 stage VI oocytes were manually sorted, treated with 4.5 μg/ml progesterone, and checked for >90% GVBD overnight. Ovaries were stored in 1× Modified Barth Saline-HEPES with 0.1 mg/ml each of penicillin and streptomycin at 18°C (Freeman *et al.*, 1989; Pickham *et al.*, 1992). For microinjections, oocytes were sorted and injected with 50 nl RNA (0.2–0.4 ng/nl). RNAs were prepared as described previously (Freeman *et al.*, 1989). Oocytes were labeled by incubation with 0.5 mCi/ml [^{35}S]Met and 0.25 mCi/ml [^{35}S]Cys, or with 20 mCi/ml [^{32}P]orthophosphate, and lysed at the indicated times before freezing on dry ice (Pickham *et al.*, 1992).

Immunoprecipitations and Kinase Assay

Oocyte lysates were clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. Supernatants were precleared with protein A-Sepharose and immunoprecipitated with the appropriate antiserum. The anti-tag polyclonal antiserum and the anti-carboxy-terminal *cdc2* rabbit antiserum were described previously (Pickham *et al.*, 1992). Immune complexes were resuspended in sample buffer and analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), or used in histone H1 kinase assays. H1 kinase assays were conducted as described elsewhere (Pickham *et al.*, 1992) and were analyzed by 12.5% SDS-PAGE and autoradiography.

Phosphopeptide Mapping and Phosphoamino Acid Analysis

Oocytes microinjected with RNAs encoding either wild-type or mutant cyclin B1 proteins were continuously labeled with [^{32}P]orthophosphate and lysed at the initiation of GVBD. Two-dimensional peptide mapping and phosphoamino acid analysis were essentially done as described previously (Luo *et al.*, 1990; Boyle *et al.*, 1991). Briefly, following immunoprecipitation and SDS-PAGE, ^{32}P-labeled cyclin B1 proteins were either eluted from gels (Boyle *et al.*, 1991) or transferred to nitrocellulose filters (Luo *et al.*, 1990). The proteins were digested with 6N HCl for phosphoamino acid analysis, or with chymotrypsin for two-dimensional phosphopeptide mapping. Peptides were first separated by electrophoresis in pH 1.9 buffer and then by ascending chromatography in phosphochromatography buffer (n-butanol, water, pyridine, and acetic acid; 75:60:50:15, vol/vol). All phosphopeptide maps were visualized by autoradiography.

One-dimensional phosphopeptide mapping was done essentially as described previously using V8 protease (Cleveland *et al.*, 1977). ^{32}P-labeled proteins were immunoprecipitated with anti-tag antiserum and separated by SDS-PAGE. The wild-type or mutant cyclin B1 bands were cut out of dried gels. The bands were rehydrated in overlay buffer (125 mM Tris-HCl, pH 6.8; 1 mM EDTA; 0.1% SDS; 1 mM 2-mercaptoethanol; 30% glycerol) for 2 min. The bands were then pushed down to the bottoms of wells in 15% SDS-PAGE. After adding 10 μl of overlay buffer above the bands, the desired amounts of V8 protease were then added above the overlay buffer. After the blue dye migrated 3–4 mm above the resolving gel, electrophoresis was stopped for 30 min to allow for protease digestion.

CSF-Arrested Egg Extracts

CSF-arrested egg extracts were prepared exactly as described elsewhere (Murray, 1991). The quality of the extracts was monitored with demembraned *Xenopus* sperm nuclei (Murray, 1991). In vitro-translated [^{35}S]Met-labeled wild-type or mutant proteins were synthesized by incubating 8 μl of nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI), 5 μl [^{35}S]Met (11 μCi/μl; NEN, Boston, MA), 1 μl RNasin (8 U/μl; Promega), and 1 μl RNA at 30°C for 1 h.

RESULTS

Epitope-Tagging and Phosphoamino Acid Analysis of Xenopus Cyclin B1

Microinjection of cyclin B1 RNA into *Xenopus* oocytes can induce oocyte maturation (Swenson *et al.*, 1986; Westendorf *et al.*, 1989; Freeman *et al.*, 1991), which facilitates mapping of cyclin B1 phosphorylation sites for the following reasons: 1) Phosphoproteins can be conveniently labeled by incubating oocytes in medium containing [^{32}P]orthophosphate. 2) Cyclin B1 proteins overexpressed in this way can be recovered from oocytes at GVBD when cyclin B1 is maximally phosphorylated, as shown in control experiments. To distinguish *endogenous* wild-type cyclin B1 from *exogenously* expressed wild-type or mutant cyclin B1, an epitope tag was incorporated at the C-terminus to allow specific recovery of cyclin B1 proteins encoded by microinjected RNAs.

As shown in Figure 1, phosphoamino acid analysis of wild-type cyclin B1, collected at GVBD, indicated

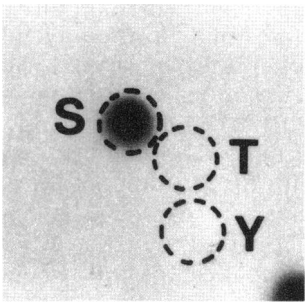

Figure 1. Phosphoamino acid analysis of cyclin B1. Thirty oocytes were microinjected with cyclin B1 RNA and labeled with [^{32}P]orthophosphate. At GVBD, oocytes were lysed and immunoprecipitated with anti-tag antiserum. ^{32}P-labeled cyclin B1 protein was recovered and subjected to phosphoamino acid analysis (see MATERIALS AND METHODS). Unlabeled phosphoamino acid markers are circled: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

that only serine residues were phosphorylated. No phosphorylation of threonine or tyrosine was detected. This result is consistent with previous reports for *Xenopus* cyclins B1 and B2 in cycling frog egg extracts, and for human cyclin B1 in HeLa cells (Pines and Hunter, 1989; Izumi and Maller, 1991; Kobayashi *et al.*, 1991a).

It should be noted that the epitope tag itself, GLEVIVVPHSLPFML, contains a single Ser residue (Pickham *et al.*, 1992; see MATERIALS AND METHODS). This raises the possibility that the epitope tag itself might introduce a new phosphorylation site. However, control experiments showed that phosphopeptide maps of wild-type cyclin B1, with or without the epitope tag, were indistinguishable. This result indicated that the serine residue within the epitope tag was not phosphorylated and, moreover, that the presence of the epitope tag at the C-terminus did not affect phosphorylation at sites within cyclin B1.

Phosphorylation Sites of Xenopus Cyclin B1

To identify serine residues phosphorylated during oocyte maturation, site-directed mutagenesis was used to construct Ser→Ala mutants at each of the 19 serine residues in *Xenopus* cyclin B1. We first screened these mutants using one-dimensional partial V8 proteolytic peptide mapping due to its simplicity and quickness. After screening all of the 19 mutant proteins in comparison with wild-type cyclin B1, we identified three mutants that clearly exhibited altered phosphopeptide patterns, these being S2A, S101A, and S113A. A typical example of a one-dimensional phosphopeptide map is presented in Figure 2, in which altered phosphopeptides are indicated for the mutants S101A and S113A (lanes 2 and 3).

To further confirm these three phosphorylation sites, we employed two-dimensional phosphopeptide mapping (Boyle *et al.*, 1991). Figure 3A shows the chymotryptic map of wild-type cyclin B1 with four major phosphopeptides as labeled. Maps displaying distinct differences are shown for three mutants: S2A (Figure 3B), S101A (Figure 3C), and S113A (Figure 3D). In each of these maps, one or more of the major phosphopeptides disappeared and, in addition, other minor changes are apparent. The disappearance of specific phosphopeptides in these Ser→Ala mutant proteins confirmed that these three serines represent phosphorylation sites. We would note that in these maps, as well as subsequent phosphopeptide maps, there are in fact more phosphopeptides than there are phosphorylation sites, and that many phosphopeptides are fainter than others. These ambiguities reflect the vagaries of phosphopeptide mapping, which often exhibits incomplete digestion products or digestion at secondary sites.

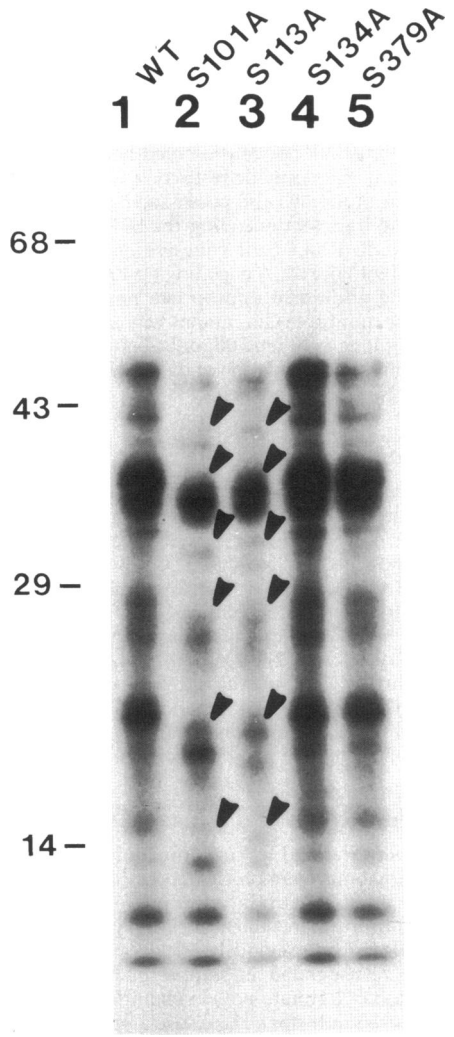

Figure 2. One-dimensional partial V8 proteolytic phosphopeptide maps. Approximately 30 oocytes were microinjected with RNAs encoding the following: lane 1, wild-type cyclin B1; lane 2, S101A; lane 3, S113A; lane 4, S134A; and lane 5, S379A. Oocytes were ^{32}P labeled as described in MATERIALS AND METHODS. At GVBD, oocytes were lysed and immunoprecipitated with anti-tag antiserum. ^{32}P-labeled wild-type or mutant cyclin B1 proteins were recovered from the immunoprecipitates by SDS-PAGE and mapped by one-dimensional phosphopeptide mapping as described in MATERIALS AND METHODS. Maps were visualized by autoradiography. Arrows indicate phosphopeptides that are missing or altered in lanes 2 and 3, compared with lane 1. Molecular weight markers are shown at the left in kilodaltons.

To investigate whether Ser2, Ser101, and Ser113 accounted for all of the serine phosphorylation of cyclin B1, we constructed the triple mutant S2A/S101A/S113A. However, as shown in Figure 5, this triple mutant (referred to as 3*A) only reduced phosphorylation by 60 to 70% (compare Figure 5, lanes 5 and 7). These results clearly demonstrated that simultaneous mutation of Ser2, Ser101, and Ser113 significantly re-

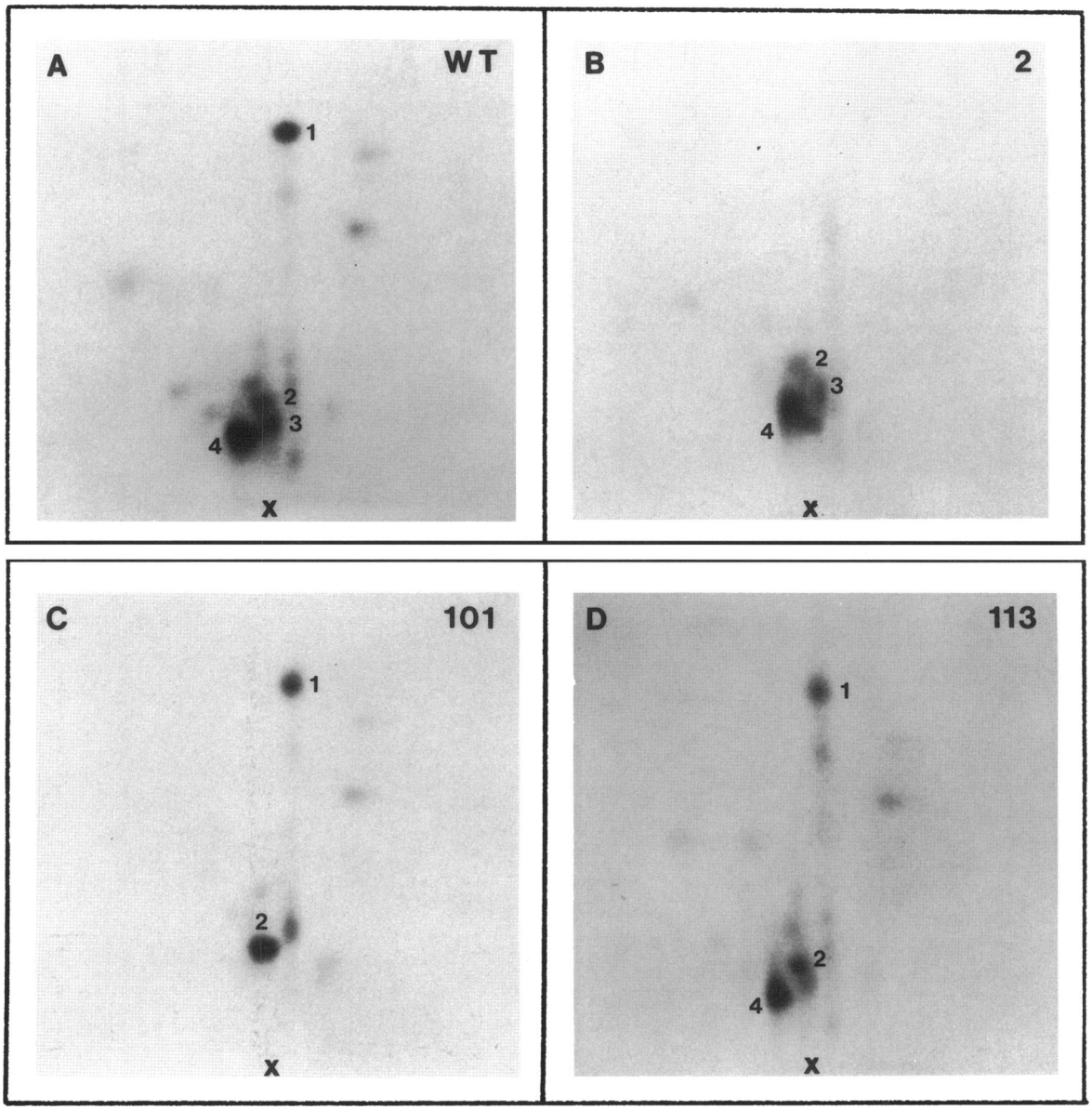

Figure 3. Two-dimensional chymotryptic phosphopeptide maps, using the method of Boyle *et al.* (1991). Approximately 100 oocytes per map were microinjected with RNA encoding the following: (A) wild-type cyclin B1; (B) S2A; (C) S101A; and (D) S113A. Major phosphopeptides are labeled with numbers as indicated. ^{32}P labeling and recovery of wild-type or mutant cyclin B1 proteins were performed as described in MATERIALS AND METHODS. Eluted proteins were oxidized with performic acid, digested with chymotrypsin, and then spotted on chromatography plates. Samples were separated by pH 1.9 electrophoresis followed by ascending chromatography. Maps were visualized by autoradiography. The origin of each map is indicated by "x." Electrophoresis was carried out in the horizontal direction, and ascending chromatography was carried out in the vertical direction.

duced phosphorylation; nonetheless, these results also indicated the existence of one or more unidentified phosphorylation sites.

To identify other phosphorylation sites, we used two-dimensional peptide mapping to screen the remaining 16 Ser→Ala single mutants, using a modified procedure that allowed faster mapping and more efficient recovery of labeled proteins (Luo *et al.*, 1990). Figure 4A presents the phosphopeptide map of wild-type cyclin B1, resolving at least seven major phosphopeptides as labeled. This map is somewhat different from that shown in Figure 3A, which was obtained using the peptide mapping procedure of Boyle (Boyle *et al.*, 1991). These differences most likely reflect differences in the order and conditions of the performic acid oxidation step and the proteolytic digestion.

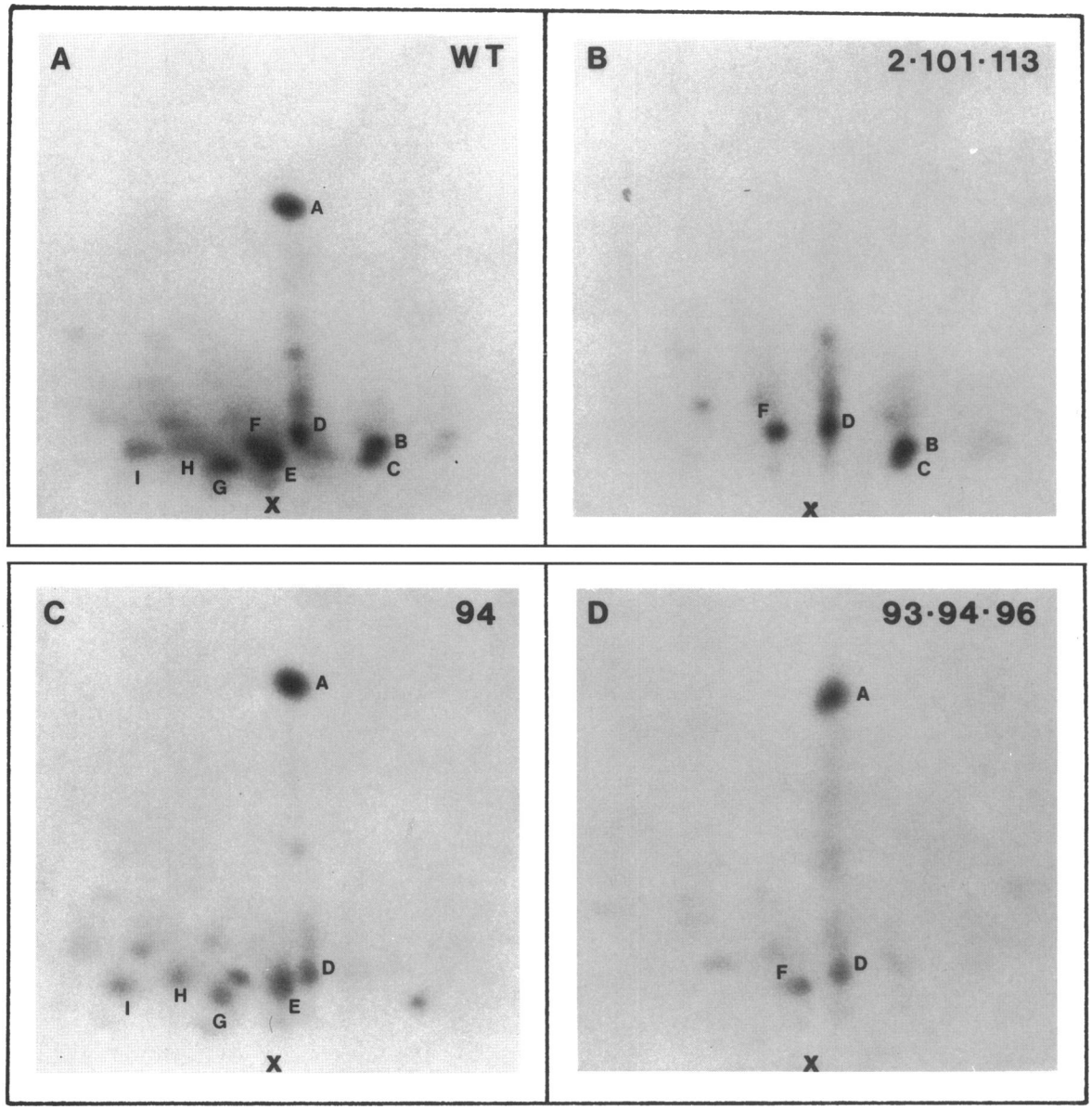

Figure 4. Two-dimensional chymotryptic phosphopeptide maps, using the method of Luo *et al.* (1990). Approximately 50 oocytes per map were microinjected with RNAs encoding the following: (A) wild-type cyclin B1; (B) S2A/S101A/S113A; (C) S94A; and (D) S93A/S94A/S96A. Because the maps prepared by this procedure are somewhat different from those in Figure 3, phosphopeptides are labeled with letters as indicated. ^{32}P labeling and purification of wild-type or mutant cyclin B1 proteins were performed as described in MATERIALS AND METHODS. Proteins were blotted to nitrocellulose, digested with chymotrypsin, then oxidized with performic acid. Two-dimensional separation was performed as in Figure 3.

Comparison of the phosphopeptide maps of wild-type cyclin B1 and the triple mutant S2A/S101A/S113A (Figure 4B) indicates that several peptides disappeared in the map of the mutant protein. Of the 16 single Ser→Ala mutant proteins examined in this way, the map of the S94A protein (Figure 4C) exhibited clear differences compared with wild-type cyclin B1, evidenced by disappearance of peptides B, C, and F. The proximity of Ser94 to other serine residues raised the possibility that there may be multiple phosphorylation sites contained within a single peptide. Therefore, we constructed the double mutant S94A/S96A, as well as the triple mutant S93A/S94A/S96A, and subjected both of these mutant proteins to peptide mapping. The resulting phosphopeptide maps for S94A/S96A and S93A/S94A/S96A (see Figure 4D) were identical, but clearly differed in comparison with the S94A and S96A single mutants. These results dem-

onstrate that both Ser94 and Ser96 represent phosphorylation sites, whereas Ser93 does not. These observations are consistent with the previous identification of Ser94 and/or Ser96 as phosphorylation sites in cyclin B1 in egg extracts (Izumi and Maller, 1991).

Several peptides did not disappear in either the map of the S2A/S101A/S113A protein or the map of the S94A/S96A protein. These phosphopeptides probably arise from the fact that some predicted chymotryptic peptides would actually contain more than one serine residue from among the five that we have identified as phosphorylation sites.

A quintuple Ala mutant was also constructed with Ser→Ala mutations at residues 2, 94, 96, 101, and 113 (referred to as 5*A). We examined phosphorylation of this quintuple Ala mutant protein as compared with wild-type cyclin B1. As shown in Figure 5, wild-type cyclin B1 was phosphorylated (Figure 5, lane 11) while no phosphorylation was detected at the position of the quintuple Ala mutant protein (Figure 5, lane 12), even though the quintuple Ala mutant protein was readily detected by ^{35}S labeling (Figure 5, compare lanes 9 and 10). This result demonstrates that there are no detectable phosphorylation sites in addition to Ser residues 2, 94, 96, 101, and 113. Note that in this and subsequent autoradiograms, the quintuple Ala mutant protein migrates slightly more rapidly in the gel than wild-type cyclin B1, presumably due to the absence of phosphorylation.

Phosphorylation of Cyclin B1 Is Not Required for cdc2 Kinase Activity or cdc2 Binding

It has been suggested that phosphorylation of cyclin B in sea urchin and also in *Xenopus* may be required for *cdc2* kinase activity (Meijer *et al.*, 1989; Gautier and Maller, 1991). Therefore, we examined immunoprecipitates containing either wild-type cyclin B1 or the quintuple Ala mutant protein for their associated H1 kinase activity, using histone H1 as an exogenous substrate. As shown in Figure 6 (lanes 3 and 4), both

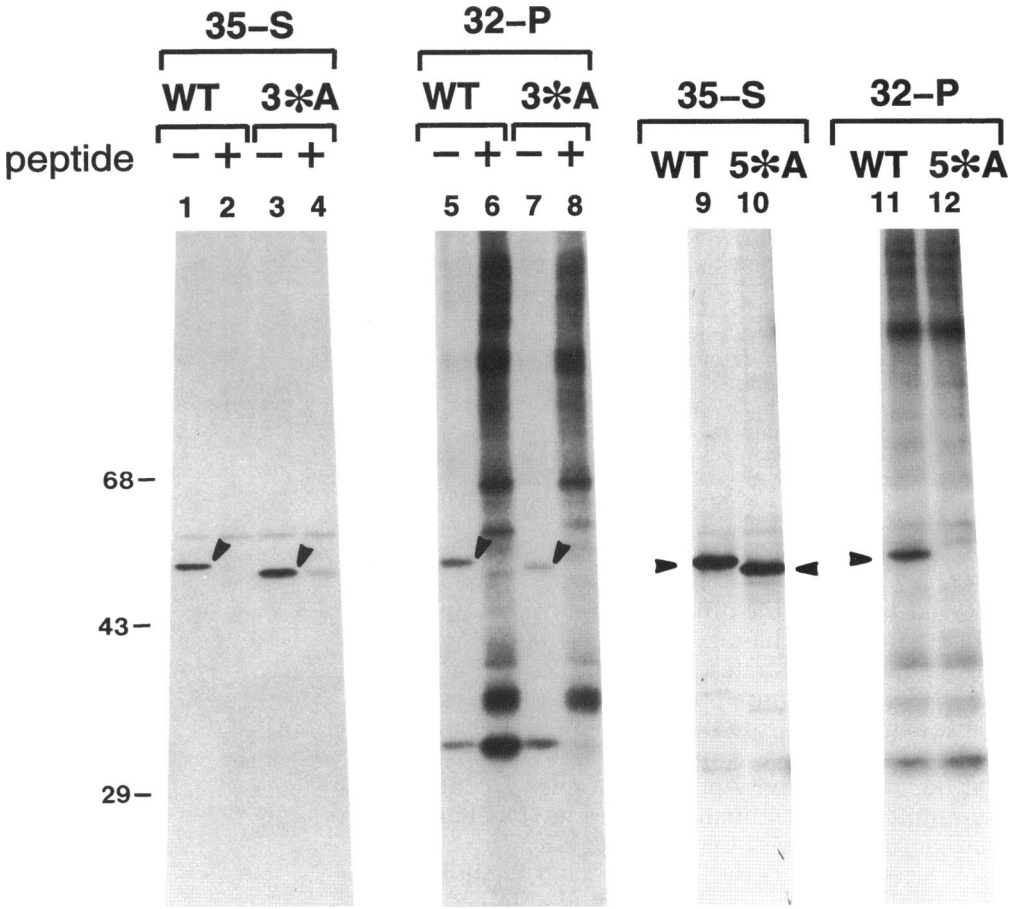

Figure 5. Phosphorylation level of wild-type and mutant cyclin B1 proteins. For each sample, microinjected oocytes were split into two groups. One group was labeled with [^{35}S]Cys and [^{35}S]Met. The other group was labeled with [^{32}P]orthophosphate. At GVBD, oocytes were lysed and immunoprecipitated with anti-tag antiserum. Samples were analyzed by 12.5% SDS-PAGE and autoradiography. For lanes 1–8, as indicated by "+" or "–", a blocking peptide was added to demonstrate the specificity of the antiserum. Lanes 1–2, ^{35}S-labeled wild-type cyclin B1; lanes 3–4, ^{35}S-labeled triple mutant protein S2A/S101A/S113A, designated "3*A"; lanes 5–6, ^{32}P-labeled wild-type cyclin B1; lanes 7–8, ^{32}P-labeled triple mutant protein S2A/S101A/S113A, designated "3*A"; lane 9, ^{35}S-labeled wild-type cyclin B1; lane 10, ^{35}S-labeled quintuple Ala mutant protein S2A/S94A/S96A/S101A/S113A, designated "5*A"; lane 11, ^{32}P-labeled wild-type cyclin B1; lane 12, ^{32}P-labeled quintuple Ala mutant protein S2A/S94A/S96A/S101A/S113A, designated "5*A". Arrows indicate bands of immunoprecipitated cyclin B1 protein. Note the absence of phosphorylated quintuple Ala mutant protein in lane 12. Molecular weight markers are indicated at the left in kilodaltons.

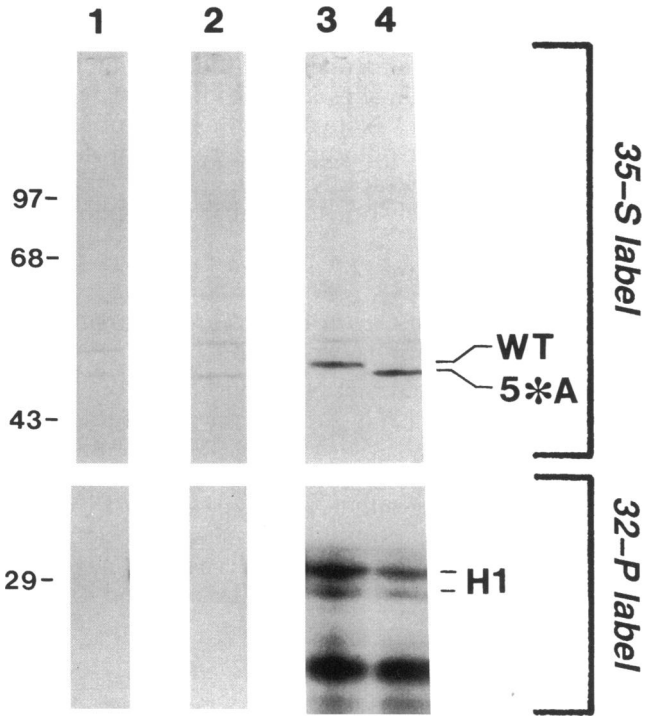

Figure 6. Histone H1 kinase activity associated with wild-type or quintuple Ala mutant cyclin B1 proteins. Oocytes were injected with RNAs encoding the following: lanes 1 and 3, wild-type cyclin B1; and lanes 2 and 4, the quintuple Ala mutant. Lanes 1 and 2 represent "blocked" immunoprecipitates due to inclusion of the cognate peptide against which the anti-tag antiserum is directed. Before immunoprecipitation, oocytes were incubated in [^{35}S]Cys and [^{35}S]Met, and at GVBD, oocytes were lysed and immunoprecipitated with anti-tag antiserum. In vitro histone H1 kinase assays were performed, using γ^{-}[^{32}P]ATP, and samples were analyzed by 12.5% SDS-PAGE and autoradiography. (Top) ^{35}S-labeled proteins were detected by direct exposure of the film to the gel. At the right, ^{35}S-labeled bands are indicated corresponding to wild-type cyclin B1 ("WT"), and the quintuple Ala mutant protein ("5*A"). (Bottom) 32P-labeled histone H1 proteins were detected using four layers of aluminum foil between the gel and the film. Note that the phosphorylated bands below 29 kDa represent breakdown products of histone H1. Molecular weight markers are shown at the left in kilodaltons.

the wild-type and the quintuple Ala mutant proteins exhibited comparable associated H1 kinase activity. This result demonstrates that phosphorylation of cyclin B1 is not required for associated *cdc2* protein kinase activity measured using histone H1 as the substrate. However, we cannot rule out the possibility that the kinase activity of MPF complexes containing the quintuple Ala mutant protein may be altered toward substrates other than histone H1.

We also compared the wild-type and the quintuple Ala mutant proteins for their ability to bind *cdc2* protein. After microinjection of RNAs encoding both cyclin B1 (quintuple Ala mutant or wild type) and *cdc2*, oocytes were labeled with [^{35}S]Met and [^{35}S]Cys. An anti-*cdc2* antiserum was then used to collect immunoprecipitates of *cdc2* protein, which were compared with immunoprecipitates collected using the anti-tag antiserum to immunoprecipitate cyclin B1 together with any *cdc2* protein complexed with it. Figure 7 reveals that the newly synthesized cyclin B1 protein is quantitatively associated with *cdc2* protein. This is expected because there is 10- to 100-fold excess of endogenous *cdc2* compared with endogenous B-type cyclins (Kobayashi *et al.*, 1991b). We conclude that phosphorylation of cyclin B1 does not alter its binding to *cdc2* protein kinase. It should be noted that the anti-*cdc2* immunoprecipitations in lanes 1 and 2 would also recover any *endogenously* expressed cyclin proteins complexed with *cdc2*. However, control experiments confirmed that these represent a quantitatively minor component compared with the *exogenously* expressed cyclin B1 proteins, as expected following microinjection of 20 ng of RNA per oocyte.

Phosphorylation of Cyclin B1 Does Not Regulate Its Turnover

Previous reports suggest that B-type cyclins are stable before the initiation of oocyte maturation (Kobayashi *et al.*, 1991b). One potential role of phosphorylation might be to regulate the stability or turnover of cyclin B1. To examine this, RNAs encoding either wild-type cyclin B1 or the quintuple Ala mutant, S2A/S94A/S96A/S101A/S113A, were microinjected into oocytes that were then labeled with [^{35}S]Met and [^{35}S]Cys.

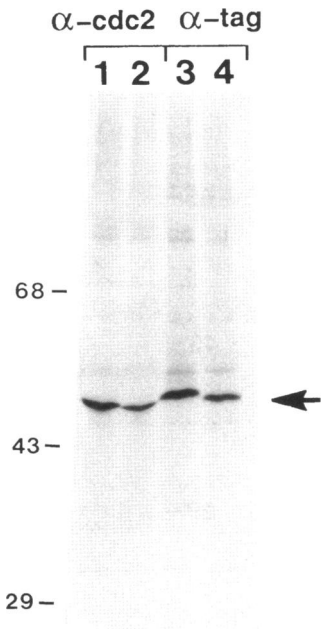

Figure 7. *Cdc2* binding to wild-type or quintuple Ala mutant cyclin B1. Oocytes were microinjected with RNAs encoding the following: lanes 1 and 3, wild-type cyclin B1; and lanes 2 and 4, the quintuple Ala mutant. Oocytes were incubated continuously in [^{35}S]Cys and [^{35}S]Met. After labeling for 3 h, oocytes were lysed and half the lysate was immunoprecipitated with anti-cdc2 antiserum (lanes 1 and 2) and the other half was immunoprecipitated with anti-tag antiserum (lanes 3 and 4). Samples were analyzed by 12.5% SDS-PAGE and autoradiography. Molecular weight markers are shown at the left in kilodaltons. Note that the bands shown in lanes 1 and 2 are somewhat distorted due to excess heavy chain Ig present in this particular antiserum migrating at a similar molecular weight.

After a 1-h labeling period, oocytes were transferred into cold medium plus 20 μg/ml cycloheximide to arrest further protein synthesis. At hourly chase intervals, labeled cyclin B1 protein was recovered by immunoprecipitation as shown in Figure 8A. This experiment indicated no significant turnover of either wild-type cyclin B1 or the quintuple Ala mutant protein during a 3-h chase period.

During oocyte maturation, cyclins B1 and B2 are partially degraded shortly after GVBD (Kobayashi *et al.*, 1991b). To examine the possible role of phosphorylation in this phenomenon, we examined the rate of turnover after GVBD for both wild-type cyclin B1 as well as the quintuple Ala mutant. For this experiment, 10 ng/oocyte of RNA encoding wild-type cyclin B1, or the quintuple Ala mutant protein, were microinjected into oocytes that were then labeled with [^{35}S]Met and [^{35}S]Cys until they underwent GVBD. To ensure that all oocytes in this experiment would mature at comparable rates, oocytes were also coinjected with a fourfold excess of *non*-epitope-tagged wild-type cyclin B1 RNA. After GVBD, oocytes were transferred into nonradioactive medium and lysed at various time intervals. The levels of the epitope-tagged cyclin B1 proteins during the chase period were determined by immunoprecipitation with the anti-tag antiserum. Figure 8B demonstrates that both wild-type cyclin B1 and the quintuple Ala mutant protein turned over after GVBD with comparable kinetics. This result demonstrates that phosphorylation is not required for the degradation of cyclin B1 after GVBD.

Destruction of B-type cyclins after fertilization is necessary for fertilized eggs to exit meiosis II (Murray *et al.*, 1989). To determine whether phosphorylation is involved in this event, we monitored turnover kinetics of cyclin B1 in a CSF-arrested egg extract. This extract mimics metaphase II-arrested eggs and can be activated by the addition of calcium (Lohka and Maller, 1985; Murray, 1991). Wild-type cyclin B1 and the quintuple Ala mutant protein were in vitro synthesized in a rabbit reticulocyte lysate and labeled with [^{35}S]Met. Subsequently, they were incubated in a CSF-arrested

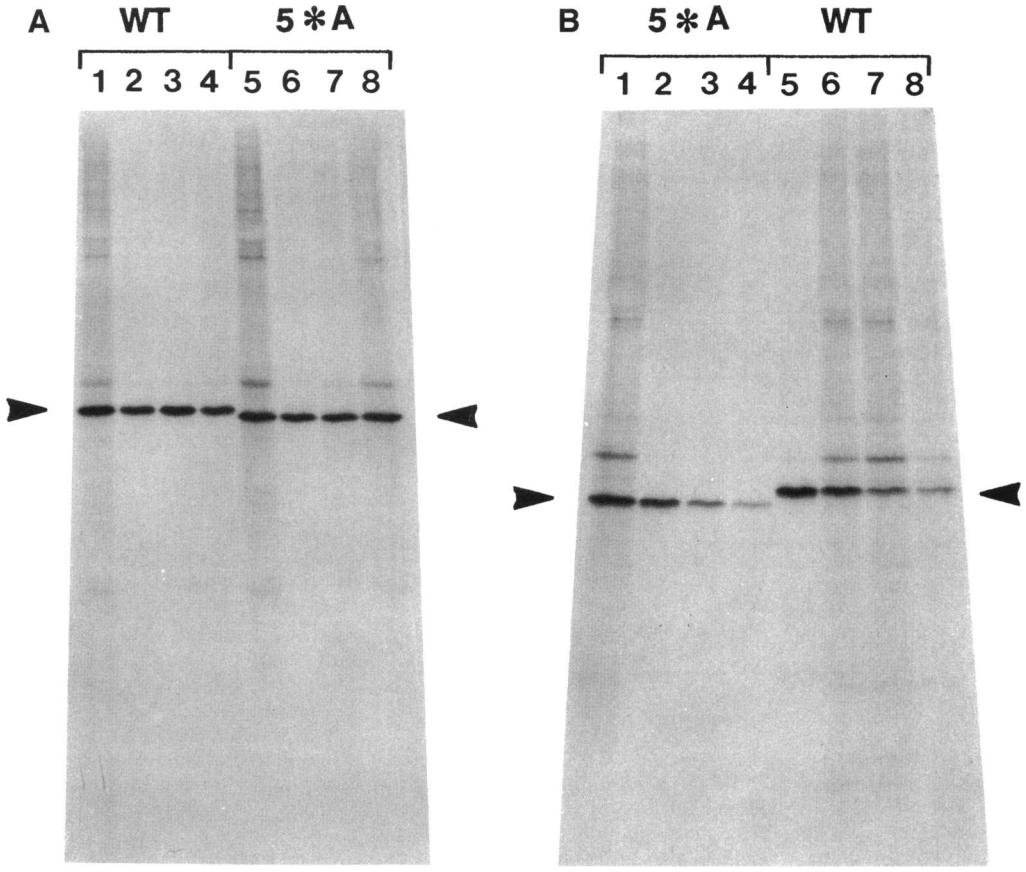

Figure 8. (A) Stability of wild-type or quintuple Ala mutant cyclin B1 in immature oocytes. Oocytes were injected with RNAs encoding the following: lanes 1–4, wild-type cyclin B1 ("WT"); and lanes 5–8, the quintuple Ala mutant ("5*A"). Oocytes were incubated in [^{35}S]Cys and [^{35}S]Met for 1 h, and then transferred into cold medium plus 20 μg/ml cycloheximide. At various chase times, oocytes were lysed and immunoprecipitated with anti-tag antiserum. Chase times were as follows: lanes 1 and 5, 0 h; lanes 2 and 6, 1 h; lanes 3 and 7, 2 h; and lanes 4 and 8, 3 h. Five oocytes were used per sample. Samples were analyzed by 12.5% SDS-PAGE and autoradiography. Arrows indicate the position of wild-type and mutant cyclin B1 proteins. (B) Degradation of wild-type or quintuple Ala mutant cyclin B1 after GVBD. Oocytes were injected with RNAs encoding the following: lanes 1–4, the quintuple Ala mutant ("5*A"); and lanes 5–8, wild-type cyclin B1 ("WT"). In this experiment, all oocytes were also injected with a fourfold excess of RNA encoding *non*-epitope-tagged wild-type cyclin B1 to allow all oocytes to mature at comparable rates. Oocytes were incubated in [^{35}S]Cys and [^{35}S]Met. At the following times after GVBD, oocytes were transferred into cold medium and lysed: lanes 1 and 5, 0 h; lanes 2 and 6, 0.5 h; lanes 3 and 7, 1 h; and lanes 4 and 8, 1.5 h. Immunoprecipitation and sample analysis were as in panel A above. Arrows indicate the position of wild-type and mutant cyclin B1 proteins.

egg extract in the presence of cycloheximide to inhibit further cyclin B synthesis. After activation of the extract with calcium, samples were withdrawn at various times and analyzed by SDS-PAGE to quantitate the amount of cyclin B1 protein remaining. As shown in Figure 9, both wild-type cyclin B1 and the quintuple Ala mutant protein turned over after calcium addition with the same kinetics. As a control, the turnover of wild-type cyclin B1 or the quintuple Ala mutant protein was shown to be relatively slow in the absence of added calcium. These results suggest that phosphorylation of the sites examined here is not required for the destruction of cyclin B1 after egg fertilization.

The Effects of Ser→Ala and Ser→Glu Mutations in Cyclin B1 on Xenopus Oocyte Maturation

It has been shown that microinjection of RNA encoding cyclin B or A into Stage VI *Xenopus* oocytes induces oocyte maturation (Swenson *et al.*, 1986; Westendorf *et al.*, 1989; Freeman *et al.*, 1991). Therefore, we used this method to assay the functional importance of cyclin B1 phosphorylation during *Xenopus* oocyte maturation. RNAs encoding the 19 Ser→Ala point mutants were individually microinjected into oocytes, which were observed for GVBD. All of the 19 mutants induced oocyte maturation as efficiently as cyclin B1, including each of the five individual phosphorylation site mutants (Table 1). Two of the mutants, S146A and S314A, required more time to reach 50% GVBD. The slow kinetics of oocyte maturation induced by mutants S146A and S314A may be explained by the location of these mutated serines within the "cyclin box" (Minshull *et al.*, 1989), which may participate in *cdc2* binding (Kobayashi *et al.*, 1992; Lees and Harlow, 1993; Zheng and Ruderman, 1993). However, these mutants were not further characterized in this work.

We also tested the double mutant S94A/S96A (2*A) and the triple mutant S2A/S101A/S113A (3*A) to see whether partial elimination of the five phosphorylation sites would interfere with oocyte maturation. Microinjection of these two RNAs into oocytes induced maturation with no detectable difference compared with wild-type cyclin B1, as shown in Figure 10, A and B (see also Table 1). Therefore, partial retention of the five phosphorylation sites is sufficient for oocyte maturation.

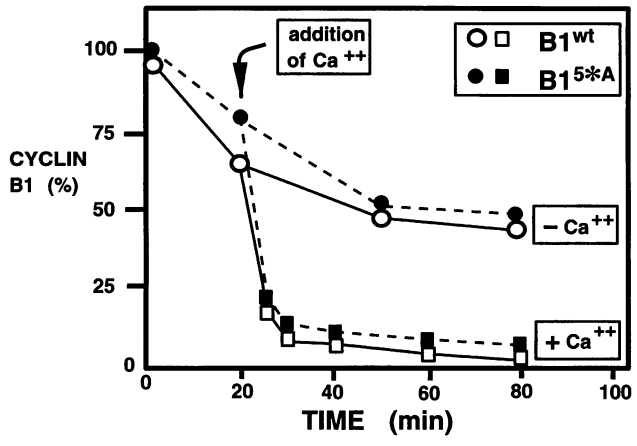

Figure 9. Destruction of wild-type or quintuple Ala mutant cyclin B1 in activated CSF-arrested egg extracts. In vitro translations (15 μl) containing [^{35}S]Met-labeled proteins, either wild type or the quintuple Ala mutant, were incubated with CSF-arrested egg extract (45 μl) containing cycloheximide at 100 μg/ml, for 20 min before the addition of 0.4 mM $CaCl_2$. Samples were taken at the indicated time points and analyzed by 12.5% SDS-PAGE and autoradiography, and then quantitated by densitometry. The quintuple Ala mutant is indicated by "5*A".

Table 1. Oocyte maturation of cyclin B1 Ser→Ala mutants

Cyclin B1 Mutant[a]	Time for 50% GVBD[b]	Final % GVBD[c]
Wild type	4.5 h	80%
S2A	4.5 h	95%
S93A	4.5 h	80%
S94A	4.0 h	85%
S96A	5.0 h	80%
S101A	4.5 h	90%
S113A	4.5 h	75%
S134A	4.0 h	85%
S146A	6.5 h	75%
S276A	3.5 h	90%
S287A	4.5 h	80%
S308A	3.5 h	90%
S314A	7.5 h	70%
S315A	4.5 h	75%
S318A	4.5 h	85%
S338A	5.0 h	90%
S372A	4.0 h	85%
S373A	4.5 h	85%
S379A	4.0 h	90%
S386A	4.0 h	90%
2*A (S→A 94, 96)	4.0 h	75%
3*A (S→A 2, 101, 113)	4.0 h	80%
5*A (S→A 2, 94, 96, 101, 113)	N/A[d]	25%
5*E (S→E 2, 94, 96, 101, 113)	4.5 h	95%

[a] Mutants indicated in bold represent mutations in phosphorylation sites, or multiple mutations in phosphorylation sites, as discussed in the text.

[b] Time required to reach 50% GVBD is reported to within the nearest half hour. For these experiments, a minimum of 15 oocytes was injected with RNA encoding each mutant, and oocytes were monitored at quarter-hour intervals for GVBD. The time required for progesterone-treated oocytes to reach 50% GVBD was approximately 7 h. All mutants were assayed in at least two individual experiments.

[c] The final percentage of oocytes to reach GVBD after overnight incubation (>15 h) is indicated (to the nearest 5%). For all experiments reported here, progesterone-treated oocytes reached at least 90% GVBD under identical conditions.

[d] N/A, not applicable, because oocytes failed to reach 50% GVBD.

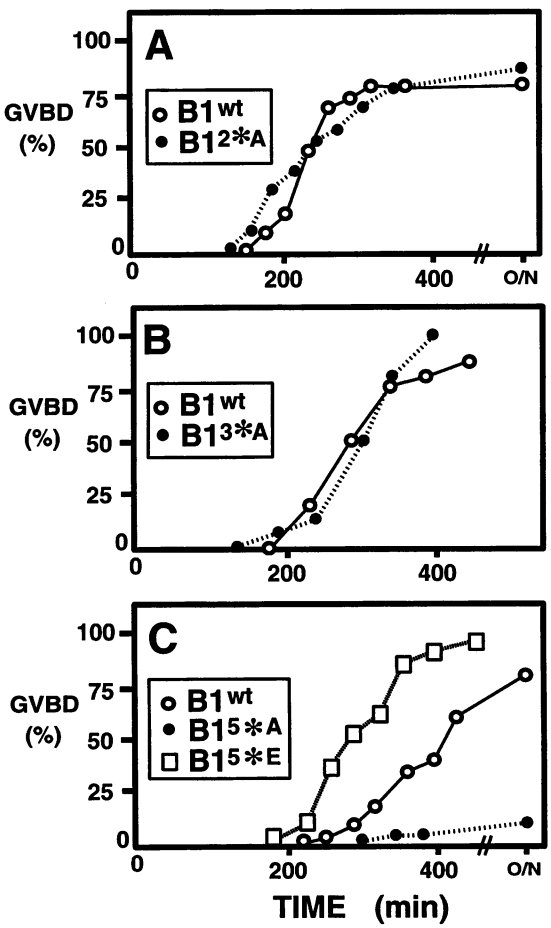

Figure 10. Time course of oocyte maturation in response to cyclin B1 mutants. Oocytes were microinjected with wild-type or mutant cyclin B1 RNAs and analyzed for GVBD. (A) Oocyte maturation is shown induced by the double mutant S94A/S96A, designated "2*A", in comparison with wild-type cyclin B1. (B) Oocyte maturation is shown induced by the triple mutant S2A/S101A/S113A, designated "3*A", in comparison with wild-type cyclin B1. (C) Oocyte maturation is shown induced by the quintuple Ala mutant S2A/S94A/S96A/S101A/S113A, designated "5*A", and by the quintuple Glu mutant S2E/S94E/S96E/S101E/S113E, designated "5*E", in comparison with wild-type cyclin B1.

When microinjected into oocytes, RNA encoding the quintuple Ala mutant S2A/S94A/S96A/S101A/S113A (5*A) did not induce oocyte maturation efficiently as compared with wild type, as shown in Figure 10C (see also Table 1). A smaller proportion of these oocytes matured, and with slower kinetics as well. These results suggest that phosphorylation of cyclin B1 is required for oocyte maturation. A quintuple Glu mutant was also constructed, with serine residues 2, 94, 96, 101, and 113 mutated to Glu (referred to as 5*E). In contrast to the very low maturation induced by the quintuple Ala mutant, the quintuple Glu mutant was able to induce oocyte maturation efficiently, and with more rapid kinetics than wild-type cyclin B1 (Figure 10C). These data confirm that phosphorylation, as mimicked by Ser→Glu mutations, confers enhanced biological activity to cyclin B1.

To further examine the enhanced biological activity of the quintuple Glu mutant, we tested the dose dependence of oocyte maturation using various amounts of RNAs encoding either wild-type cyclin B1, the quintuple Ala mutant, or the quintuple Glu mutant. These experiments showed that the quintuple Glu mutant consistently stimulated more efficient oocyte maturation than wild-type cyclin B1 at all concentrations assayed from 0.5–30 ng of microinjected RNA per oocyte. For each RNA, maximal oocyte maturation was induced by 5–10 ng per oocyte; above this amount, our observations show that no greater maturation was achieved.

We also wished to examine whether the quintuple Ala mutant might function as a dominant negative inhibitor of oocyte maturation. This was examined as shown in Figure 11 by coinjection of RNAs encoding either wild-type cyclin B1 or the quintuple Glu mutant, alone or in combination with the quintuple Ala mutant. By itself, expression of the quintuple Ala mutant induced a low level of oocyte maturation (19% GVBD), whereas efficient oocyte maturation was induced by wild-type cyclin B1 (75% GVBD) or by the quintuple Glu mutant (94% GVBD). Coinjection of the quintuple Ala mutant had negligible effects on oocyte maturation induced by either wild-type cyclin or by the quintuple Glu mutant. This suggests that the ab-

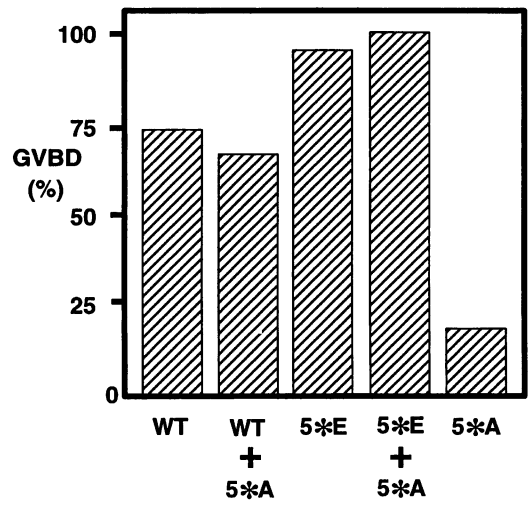

Figure 11. Oocyte maturation induced by coinjection of RNAs encoding wild-type cyclin B1, the quintuple Ala mutant, or the quintuple Glu mutant. Each RNA sample was injected at 10 ng/oocyte, and maturation was scored as indicated by the percentage of oocytes that reached GVBD. The quintuple Ala mutant S2A/S94A/S96A/S101A/S113A is designated as "5*A". The quintuple Glu mutant S2E/S94E/S96E/S101E/S113E is designated as "5*E".

sence of phosphorylation leads to an inactive, but not an inhibitory, cyclin B1 protein.

DISCUSSION

In this work, we have used the indirect approach of site-directed mutagenesis in combination with phosphopeptide mapping to identify Ser2, Ser94, Ser96, Ser101, and Ser113 as presumptive phosphorylation sites in *Xenopus* cyclin B1. Elimination of these sites, either singly or in various combinations of Ser→Ala mutations, leads to the disappearance of all observed phosphopeptides, and there is no detectable ^{32}P labeling of the quintuple Ala mutant protein. Phosphorylation of cyclin B1 does not appear to be required for *cdc2* kinase activity, for binding of cyclin B1 to *cdc2*, for stability of cyclin B1 before GVBD, or for degradation of cyclin B1 after GVBD or after egg activation. At least partial phosphorylation of cyclin B1 appears to be required for *Xenopus* oocyte maturation, as demonstrated by the undiminished activity of mutants that only partially remove the phosphorylation sites (see Table 1), in contrast to the dramatically reduced activity of the quintuple Ala mutant. Moreover, the quintuple Glu mutant, designed to mimic phosphorylated cyclin B1, induced efficient oocyte maturation with more rapid kinetics than wild-type cyclin B1.

Phosphorylation of Cyclin B1

Of the five phosphorylation sites, four are located within a 20-amino acid stretch from amino acid 94 to amino acid 113. This region is highly conserved among B-type cyclins from vertebrates, although it is not conserved among invertebrates. Although we have identified all phosphorylation sites that are utilized during oocyte maturation up to the time of GVBD, we have no data on whether there exists a temporal order of phosphorylation at the different sites or, in fact, whether all sites are phosphorylated on a single protein molecule. Noticeable mobility differences are evident between wild-type and the quintuple Ala mutant proteins as soon as 45 min after microinjection, suggesting that phosphorylation of cyclin B1 is a very early event in meiotic maturation. Phosphorylation of *Xenopus* cyclin B2 was previously reported in resting oocytes (Gautier and Maller, 1991). We have no data concerning the identity of the protein kinases that may be involved in serine phosphorylation of cyclin B1 at specific sites, although Izumi and Maller (1991) previously showed that MPF and MAP kinase can phosphorylate cyclin B1 at Ser94 and/or 96. Clearly, the identification of kinases(s) responsible for in vivo phosphorylation of cyclin B1 will be an area of future interest.

Our data indicate that phosphorylation of cyclin B1 is not required for binding to *cdc2*. Consistent with this, the *cdc2* binding domain in cyclin A has been mapped (Kobayashi *et al.*, 1992; Lees and Harlow, 1993) and the phosphorylation sites of cyclin B1 are located outside this region. We also showed that phosphorylation of cyclin B1 is not required for *cdc2* kinase activity assayed using histone H1 as the substrate. This is evidenced by the fact that the *cdc2* kinase activity associated with the quintuple Ala mutant protein is comparable to that associated with wild-type B1.

Requirement of Phosphorylation of Cyclin B1 for Oocyte Maturation

Exactly how the expression of exogenous cyclin B1 induces oocyte maturation is unclear. One possible mechanism might require that B-type cyclin accumulates to a threshold level which, once attained, leads directly or indirectly to the suppression of *wee1* kinase and the activation of *cdc25*. This may simply require that sufficient B-type cyclin accumulates such that the resulting pre-MPF complexes overcome the inhibitory activity of *wee1*, leading to the autoactivation of the remaining pre-MPF.

In this work, we have shown that the quintuple Ala mutant induces oocyte maturation much less efficiently than wild-type cyclin B1, whereas the quintuple Glu mutant induces oocyte maturation even more efficiently than wild type. Although it seems clear that phosphorylation of cyclin B1 regulates its biological activity, the molecular mechanism of this regulation is unclear. Previous work has discussed the possibility that phosphorylation may regulate the targeting of cyclin B1 complexes to appropriate subcellular locations (Pines and Hunter, 1991; Gallant and Nigg, 1992). Recent work by Pines and Hunter (1994) has demonstrated that human cyclins B1 and A are differentially localized to the nucleus, with cyclin B1 localizing primarily to the cytoplasm. A putative "cytoplasmic retention signal" in cyclin B1 has been identified that includes the phosphorylation sites at residues 94, 96, 101, and 113, suggesting that phosphorylation may regulate the differential subcellular localization of cyclin proteins. We are currently examining this possibility. Phosphorylation of cyclin B1 may also be required for MPF to phosphorylate specific targets involved in GVBD, such as nuclear lamins that are reportedly phosphorylated by MPF to dissolve the nuclear envelope (Heald and McKeon, 1990; Peter *et al.*, 1990; Ward and Kirschner, 1990). The elucidation of specific cyclin functions mediated by phosphorylation will contribute greatly to our understanding of cell cycle regulation.

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