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Mutants at Ser277 of *Xenopus* cdc2 Protein Kinase Induce Oocyte Maturation in the Absence of the Positive Regulatory Phosphorylation Site Thr161

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The *cdc2* protein kinase is an important regulatory protein for both meiosis and mitosis. Previously, we demonstrated that simultaneous mutation of $Thr^{14} \rightarrow Ala^{14}$ and $Tyr^{15} \rightarrow Phe^{15}$ in the *Xenopus cdc2* protein results in an activated *cdc2* mutant that induces maturation in resting oocytes. In addition, we confirmed the importance of the positive regulatory phosphorylation site, Thr^{161}, by demonstrating that *cdc2* mutants containing additional mutations of $Thr^{161} \rightarrow Ala^{161}$ or Glu^{161} are inactive in the induction of oocyte maturation. Here, we have analyzed the importance of an additional putative *cdc2* phosphorylation site, Ser^{277}. Single mutation of $Ser^{277} \rightarrow Asp^{277}$ or Ala^{277} had no effect on activity, and these mutants were unable to induce *Xenopus* oocyte maturation. However, the double mutant Ala^{161}/Asp^{277} was capable of inducing oocyte maturation, suggesting that mutation of $Ser^{277} \rightarrow Asp^{277}$ could compensate for the mutation of $Thr^{161} \rightarrow Ala^{161}$. The Asp^{277} mutation could also compensate for the Ala^{161} mutation in the background of the activating mutations Ala^{14}/Phe^{15}. Although mutants containing the compensatory Ala^{161} and Asp^{277} mutations were capable of inducing oocyte maturation, these mutant *cdc2* proteins lacked detectable in vitro kinase activity. Tryptic phosphopeptide mapping of mutant *cdc2* protein and comparison with in vitro synthesized peptides indicated that Ser^{277} is not a major site of phosphorylation in *Xenopus* oocytes; however, we cannot rule out the possibility of phosphorylation at this site in a biologically active subpopulation of *cdc2* molecules. The data presented here, together with prior reports of Ser^{277} phosphorylation in somatic cells, suggest an important role for Ser^{277} in the regulation of *cdc2* activity. The regulatory role of Ser^{277} most likely involves its indirect effects on the nearby residue Arg^{275}, which participates in a structurally important ion pair with Glu^{173}, which lies in the same loop as Thr^{161} in the *cdc2* protein.

INTRODUCTION

The *cdc2* protein kinase is a component of maturation promoting factor (MPF), an important cell cycle regulatory complex. MPF consists of two subunits, the *cdc2* protein kinase and a cyclin protein (Dunphy *et al.*, 1988; Gautier *et al.*, 1988). 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 (Maller, 1990; Nurse, 1990; Pines and Hunter, 1990; Lew *et al.*, 1991; Matsushime *et al.*, 1992; Meyerson *et al.*, 1992). These *cdc2* and cyclin homologues can combine to form various complexes that are active in the G2/M and/or the G1/S stages of the cell cycle. The *cdc2* protein has been shown to contain three regulatory phosphorylation sites. These are Thr^{14} and Tyr^{15}, both of which play a negative regulatory role, and Thr^{161},

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which plays a positive regulatory role (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Krek and Nigg, 1991a,b; Norbury *et al.*, 1991; Pickham *et al.*, 1992; Solomon *et al.*, 1992). Previously, we demonstrated that mutation of $Thr^{14} \rightarrow Ala^{14}$ and $Tyr^{15} \rightarrow Phe^{15}$ in *Xenopus cdc2* results in an activated mutant that induces maturation of resting oocytes (Pickham *et al.*, 1992).

Thr^{161} is located in a region that is conserved among protein kinases (Hanks *et al.*, 1988), and mutations at this site can produce a variety of phenotypes, including inactivity, lethality, or dominant negativity, depending upon the substituted amino acid and the specific assay system (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Solomon *et al.*, 1992; Pickham *et al.*, 1992). Phosphorylation of Thr^{161} is clearly linked to its role in cyclin binding, as $Thr^{161} \rightarrow Ala^{161}$ mutants show a decreased affinity for cyclin (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Pickham *et al.*, 1992; Marcote *et al.*, 1993). A kinase that phosphorylates Thr^{161} has recently been isolated from both *Xenopus* eggs and star fish oocytes and found to be identical to a previously characterized maternal RNA in *Xenopus* known as MO15 (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993). Whereas phosphorylation of Thr^{161} is critical for function, there also appears to be an important role for dephosphorylation of Thr^{161} in inactivation of *cdc2* and exit from mitosis (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Lorca *et al.*, 1992). The dephosphorylation of *cdc2* involves protein phosphatase 2A (PP2A), as PP2A itself or an extract from *Xenopus* oocytes containing a PP2A related molecule has been shown to efficiently dephosphorylate Thr 161 in vitro (Kinoshita *et al.*, 1990; Gould *et al.*, 1991; Lee *et al.*, 1991).

Xenopus oocyte maturation, which requires progression past G2 of the first meiotic division, has been shown to be regulated by MPF, which can be activated either by increasing amounts of cyclin or by the dephosphorylation of Thr^{14} and Tyr^{15} of *cdc2* (Swenson *et al.*, 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989; Tachibana *et al.*, 1990; Freeman *et al.*, 1991; Gautier *et al.*, 1991; Lee *et al.*, 1992). Our previous study of *cdc2* mutants suggested a critical role for Thr^{161} in the completion of meiosis during *Xenopus* oocyte maturation (Pickham *et al.*, 1992). In contrast to the clear regulatory role of threonine phosphorylation, there is no evidence that serine phosphorylation of *cdc2* plays a regulatory role during oocyte maturation, even though multiple sites of serine phosphorylation were observed on *cdc2* isolated from *Xenopus* oocytes (Gabrielli *et al.*, 1992). In cycling chicken cells, undergoing mitosis rather than meiosis, Ser^{277} has been identified as a site of phosphorylation (Krek and Nigg, 1991a). Nonetheless, no serine phosphorylation was detected on *cdc2* in *Xenopus* egg extracts undergoing mitotic cycling (Solomon *et al.*, 1992). Thus, the potential importance of serine phosphorylation in the regulation of *cdc2* activity remains unresolved.

In this work, we wished to examine the potential regulatory role of Ser^{277} during *Xenopus* oocyte maturation. We constructed mutations of Ser^{277} to both Ala^{277} and Asp^{277} and also recombined these mutations with previously described mutations affecting Thr^{14}, Tyr^{15}, or Thr^{161}. Surprisingly, we discovered that mutation of $Ser^{277} \rightarrow Asp^{277}$ could restore biological activity to mutant *cdc2* proteins that were inactivated by mutation of $Thr^{161} \rightarrow Ala^{161}$. It is clear, then, that this site has a potentially important role in the regulation of *cdc2* activity.

MATERIALS AND METHODS

Mutagenesis of Ser^{277}

Single-stranded *Xenopus cdc2* DNA was isolated and mutagenized as described (Kunkel, 1985) using the following oligonucleotides: $Ser^{277} \rightarrow Ala^{277}$, CCCGCCAAGAGGATTGCCGCCCGAAAGGCTATGCTGC, and $Ser^{277} \rightarrow Asp^{277}$, GATCCCGCCAAGAGGATCGATGCACGAAAAGCTATGC. After mutagenesis, restriction fragments containing the mutated sequence were sequenced and then ligated into the corresponding region of wild-type *Xenopus cdc2* in pSP64(polyA) (Pickham *et al.*, 1992). Plasmids containing *cdc2* genes with multiple mutations were made by replacing the wild-type sequence with restriction fragments containing the desired mutation from plasmids containing previously characterized *cdc2* mutants. Epitope-tagged derivatives of *cdc2*, referred to as *cdc2*-TAG, contain an epitope tag derived from the cytoplasmic domain of the platelet-derived growth factor receptor as described previously (Pickham *et al.*, 1992).

Oocyte Microinjection

Adult female *Xenopus* were obtained from *Xenopus* I (Ann Arbor, MI). Frogs were primed with 50 IU pregnant mare serum gonadotropin (Calbiochem-Behring, San Diego, CA) 3 d before use. Oocytes were sorted manually and stored in MBS-H (10 mM *N*-2-hydroethylpiperazine-*N'*-2-ethanesulfonic acid pH 7.4, 88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca[No_3]_2$) with 0.1 mg/ml each penicillin and streptomycin at 18°C for 4–18 h before use, as previously described (Freeman *et al.*, 1989). Oocytes were microinjected with 50 nl RNA (2 mg/ml). Oocytes were labeled with 0.5 mCi/ml [^{35}S]Cys and [^{35}S]Met or 20 mCi/ml [^{32}P]orthophosphate and lysed in cold lysis buffer (Pickham *et al.*, 1992) at the indicated times and frozen on dry ice or used immediately for immunoprecipitations.

Immunoprecipitations and Kinase Assays

Samples 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-epitope-tag polyclonal antiserum, referred to as anti-TAG serum, and the anti-carboxy-terminal *cdc2* rabbit antisera 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 previously (Pickham *et al.*, 1992) and were analyzed by 12.5% SDS-PAGE and autoradiography.

Peptide Mapping and Phosphoamino Acid Analysis

Oocytes expressing *cdc2*-TAG proteins for peptide mapping were continuously labeled with [^{32}P]orthophosphate from 0–4 h after microinjection of in vitro–transcribed RNAs and were lysed at 4 h. In

control experiments, essentially identical tryptic phosphopeptide maps were obtained for *cdc2* proteins recovered at either 4 h or at $GVBD_{50}$, in each case with continuous [^{32}P]labeling after microinjection.

Peptide mapping was essentially as described previously (Luo *et al.*, 1990; Boyle *et al.*, 1991). Briefly, after immunoprecipitation and SDS-PAGE, [^{32}P]-labeled samples were transferred to nitrocellulose filters. Bands containing *cdc2* protein were eluted and digested with trypsin, and the resulting peptides were resuspended in pH 1.9 buffer. Peptides were separated first 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]).

For experiments with synthetic peptides, the ISAR peptide was synthesized by Dr. Elizabeth Komives at the University of California, San Diego (UCSD), and the 794 peptide (YDPAKRISARKALL) was synthesized by the UCSD Peptide and Oligonucleotide Facility. Peptides were phosphorylated in vitro by the H87A mutant of protein kinase A (PKA) that exhibits relaxed substrate specificity, generously provided by Dr. Susan S. Taylor, UCSD. Reactions were done in 0.1 M 3-(*N*-morpholino)propanesulfonic acid pH 7.0, 1 mM $MgCl_2$, 5 μM ATP, 100 μg/ml peptide, 10 μCi γ-[^{32}P]ATP, 0.1 μg/ml mutant PKA in a final volume of 30 μl for 20 min at 30°C. The phosphorylated peptides were purified by electrophoresis and chromatography as described above and were eluted from the cellulose with pH 1.9 buffer and lyophilized. The 794 peptide was then subjected to trypsin digestion and a second round of purification. The phosphorylated peptides were analyzed for phosphoamino acid content as described (Boyle *et al.*, 1991) and found to contain only phosphoserine.

Table 1. Biological activity of *cdc2* mutants

cdc2 mutant	# oocytes[a]	% GVBD	Induction of oocyte maturation[b]
$T^{14} \cdot Y^{15} \cdot T^{161} \cdot S^{277}$ [c]	105	0	−
$T^{14} \cdot Y^{15} \cdot T^{161} \cdot \mathbf{A^{277}}$	37	0	−
$T^{14} \cdot Y^{15} \cdot T^{161} \cdot \mathbf{D^{277}}$	22	0	−
$T^{14} \cdot Y^{15} \cdot \mathbf{A^{161}} \cdot S^{277}$	[d]	0	−
$T^{14} \cdot Y^{15} \cdot \mathbf{A^{161}} \cdot \mathbf{D^{277}}$	25	56	+
$\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot T^{161} \cdot S^{277}$	130	62	+
$\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot T^{161} \cdot \mathbf{A^{277}}$	130	68	+
$\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot T^{161} \cdot \mathbf{D^{277}}$	37	0	−
$\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot \mathbf{A^{161}} \cdot S^{277}$	[d]	0	−
$\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot \mathbf{A^{161}} \cdot \mathbf{A^{277}}$	37	0	−
$\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot \mathbf{A^{161}} \cdot \mathbf{D^{277}}$	52	55	+
$\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot \mathbf{E^{161}} \cdot \mathbf{A^{277}}$	37	0	−
$\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot \mathbf{E^{161}} \cdot \mathbf{D^{277}}$	36	0	−
Progesterone	52	80	+

Mutant residues are shown in bold type.

[a] Data shown represent totals from two to four separate experiments for each mutant.

[b] + indicates oocyte maturation, whereas − indicates no oocyte maturation.

[c] Wild-type *cdc2* has the genotype $T^{14} \cdot Y^{15} \cdot T^{161} \cdot S^{277}$.

[d] Data for these mutants previously reported (Pickham *et al.*, 1992).

RESULTS

Convention for Nomenclature of Mutants

In this work, the new mutations of $Ser^{277} \rightarrow Ala^{277}$ or $Ser^{277} \rightarrow Asp^{277}$ have been combined with previously described mutants (Pickham *et al.*, 1992), resulting in several triple and quadruple mutants. These mutants are listed in Table 1. To avoid confusion, the names of mutants will always refer to each of the four following residues, whether wild-type or mutant: #14 (Thr or Ala), #15 (Tyr or Phe), #161 (Thr, Ala, or Glu), and #277 (Ser, Ala, or Asp). Single letter abbreviations for the amino acids will be used in these names, and any mutant residues will be denoted by bold type whereas wild-type residues will be denoted by regular type. Thus, the wild-type *cdc2*, $Thr^{14}/Tyr^{15}/Thr^{161}/Ser^{277}$, will be denoted $T^{14} \cdot Y^{15} \cdot T^{161} \cdot S^{277}$. The quadruple mutant, $Ala^{14}/Phe^{15}/Ala^{161}/Asp^{277}$, will be denoted $\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot \mathbf{A^{161}} \cdot \mathbf{D^{277}}$. As a last example, the *cdc2* mutant with a single mutation of $Ser^{277} \rightarrow Ala^{277}$, which therefore contains the wild-type residues Thr^{14}, Tyr^{15}, and Thr^{161}, will be denoted $T^{14} \cdot Y^{15} \cdot T^{161} \cdot \mathbf{A^{277}}$.

Role of Ser^{277} in cdc2 Activity

Previously, a Ala^{14}/Phe^{15} double mutant was shown to induce GVBD in resting oocytes (Pickham *et al.*, 1992). As *cdc2* in chicken fibroblasts is phosphorylated on Ser^{277} (Krek and Nigg, 1991a), we decided to investigate the importance of Ser^{277} as a potential regulatory site in *Xenopus cdc2*. We therefore constructed mutations of $Ser^{277} \rightarrow Ala^{277}$ or Asp^{277} in *Xenopus cdc2* and recombined them with previously described mutations to yield the mutants shown in Table 1. For each mutant, in vitro–synthesized RNA was prepared and microinjected into resting Stage VI *Xenopus* oocytes, which were then observed and scored for maturation as indicated by GVBD.

The results of these experiments, shown in Table 1, indicated that mutation of $Ser^{277} \rightarrow Ala^{277}$ had no effect on the activity of the *cdc2* mutant, regardless of the genetic background. Thus, the wild-type *cdc2* protein, $T^{14} \cdot Y^{15} \cdot T^{161} \cdot S^{277}$, was unable to induce oocyte maturation and so was the $Ser^{277} \rightarrow Ala^{277}$ derivative, $T^{14} \cdot Y^{15} \cdot T^{161} \cdot \mathbf{A^{277}}$. Similarly, the biological activity of the activated double mutant, $\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot T^{161} \cdot S^{277}$, which can induce oocyte maturation, was unchanged by mutation of $Ser^{277} \rightarrow Ala^{277}$ as in the mutant $\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot T^{161} \cdot \mathbf{A^{277}}$.

Mutation of $Ser^{277} \rightarrow Asp^{277}$ was undertaken to introduce a negative charge and potentially mimic a phosphoserine residue. Table 1 shows that mutation of $Ser^{277} \rightarrow Asp^{277}$, when present with the wild-type residue Thr^{161}, always resulted in an inactive *cdc2* mutant. This was true whether the parental phenotype was inactive or active in oocyte maturation assays. For instance, the wild-type *cdc2* protein, $T^{14} \cdot Y^{15} \cdot T^{161} \cdot S^{277}$, was unable to induce oocyte maturation and so was the $Ser^{277} \rightarrow Asp^{277}$ derivative, $T^{14} \cdot Y^{15} \cdot T^{161} \cdot \mathbf{D^{277}}$. In contrast, the double mutant, $\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot T^{161} \cdot S^{277}$, can induce oocyte maturation, but this activity was lost when the $Ser^{277} \rightarrow Asp^{277}$ mutant was introduced to create the $\mathbf{A^{14}} \cdot \mathbf{F^{15}} \cdot T^{161} \cdot \mathbf{D^{277}}$ mutant.

Surprisingly, a third conclusion emerges from the data presented in Table 1, which shows that the mutation

$Ser^{277} \rightarrow Asp^{277}$ exerted very unexpected effects when assayed in conjunction with the mutation $Thr^{161} \rightarrow Ala^{161}$, which removes the site of positive regulatory phosphorylation. For example, we showed previously (Pickham *et al.*, 1992) that the single mutant $T^{14} \cdot Y^{15} \cdot \boldsymbol{A^{161}} \cdot S^{277}$ is inactive for oocyte maturation. However, this mutant became active after introduction of the $Ser^{277} \rightarrow Asp^{277}$ mutation to create the $T^{14} \cdot Y^{15} \cdot \boldsymbol{A^{161}} \cdot \boldsymbol{D^{277}}$ mutant. Also, we previously described the inactive triple mutant $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot \boldsymbol{A^{161}} \cdot S^{277}$, which contains the activating mutations Ala^{14} and Phe^{15}, but lacks Thr^{161}. This mutant also became active for induction of oocyte maturation after introduction of the $Ser^{277} \rightarrow Asp^{277}$ mutation to create the quadruple mutant $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot \boldsymbol{A^{161}} \cdot \boldsymbol{D^{277}}$. This effect clearly required the presence of the negatively charged Asp residue, as mutation of $Ser^{277} \rightarrow Ala^{277}$ had no effect, as shown by the inactivity of the quadruple mutant $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot \boldsymbol{A^{161}} \cdot \boldsymbol{A^{277}}$. These results demonstrate that the loss of the positive regulatory phosphorylation site Thr^{161} can be partially compensated by mutation of $Ser^{277} \rightarrow Asp^{277}$.

Table 1 also presents results obtained with mutants containing the $Thr^{161} \rightarrow Glu^{161}$ mutation, described previously (Pickham *et al.*, 1992). These mutants were inactive and could not be reactivated by mutation of $Ser^{277} \rightarrow Ala^{277}$ or Asp^{277}. This is evidenced by the inactivity of the quadruple mutants $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot \boldsymbol{E^{161}} \cdot \boldsymbol{A^{277}}$ and $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot \boldsymbol{E^{161}} \cdot \boldsymbol{D^{277}}$.

To allow specific recovery of each of these proteins from oocytes, an epitope tag was introduced at the carboxy-terminus of the mutant *cdc2* proteins. This epitope tag allows specific recovery of the mutant *cdc2*-TAG proteins resulting from translation of microinjected RNA (Pickham *et al.*, 1992). RNA encoding each mutant *cdc2* was injected into oocytes that were metabolically labeled; mutant *cdc2* proteins were then immunoprecipitated with anti-TAG antiserum and analyzed by SDS-PAGE and fluorography. Oocytes injected with RNAs encoding each of the mutants synthesized a 34-kDa protein that could be immunoprecipitated with a specific anti-TAG antiserum (Figure 1). This demonstrates that the *cdc2* mutant proteins are all synthesized in vivo. Several of the *cdc2* proteins display a doublet in Figure 1, previously observed (Pickham *et al.*, 1992), which may reflect changes in the phosphorylation and/or conformation of *cdc2* during oocyte maturation. The proteins exhibiting this phenotype include the wild-type (lane 4), the $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot T^{161} \cdot \boldsymbol{A^{277}}$ mutant (lane 5), and to a lesser extent the $T^{14} \cdot Y^{15} \cdot T^{161} \cdot \boldsymbol{A^{277}}$ mutant (lane 6) and the $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot T^{161} \cdot \boldsymbol{D^{277}}$ mutant (lane 9).

Previously, we demonstrated that mutations at Thr^{161} significantly reduced the ability of mutant *cdc2* proteins to bind cyclin B1 in coimmunoprecipitation experiments, exhibiting only ~25% as much cyclin binding as wild-type *cdc2* (Pickham *et al.*, 1992). In attempting to understand the biological activity of the $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot \boldsymbol{A^{161}} \cdot \boldsymbol{D^{277}}$ and $T^{14} \cdot Y^{15} \cdot \boldsymbol{A^{161}} \cdot \boldsymbol{D^{277}}$ mutants, we

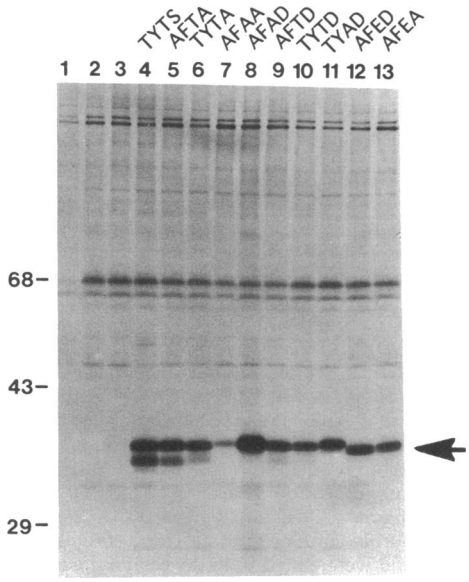

Figure 1. Recovery of *cdc2* proteins labeled in vivo. Oocytes were treated as follows: lane 1, treated with progesterone; lane 2, untreated; lane 3, injected with water as a control; lanes 4–13, injected with in vitro–transcribed *cdc2*-TAG RNAs. Oocytes were labeled with [^{35}S]Cys and [^{35}S]Met for 6 h, then lysed and immunoprecipitated with anti-TAG antiserum. The arrow indicates the position of the *cdc2*-TAG protein. Samples were analyzed by 12.5% SDS-PAGE and fluorography.

investigated whether the Asp^{277} mutation would restore wild-type cyclin binding to mutant *cdc2* proteins carrying the Ala^{161} mutation. The anti-TAG antiserum was used to recover mutant *cdc2*-TAG proteins from oocytes that were also overexpressing cyclin B1. The metabolically labeled proteins were then analyzed by SDS-PAGE, and the ratio of cyclin:*cdc2* was quantitated as described previously (Pickham *et al.*, 1992). We found that the presence of the Asp^{277} mutation resulted in no significant increase in the amount of cyclin B1 protein that could be coimmunoprecipitated with mutant *cdc2* proteins carrying the Ala^{161} mutation. This indicates that the Asp^{277} mutation does not compensate for the Ala^{161} mutation by restoring the ability to bind cyclin B1 protein.

Rate of Maturation Induced by Mutants at Ser^{277}

Each of the *cdc2*-TAG mutant RNAs was analyzed for the rate of induction of GVBD. Previously, it was shown that the $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot T^{161} \cdot S^{277}$ mutant induces GVBD at a slower rate than progesterone or mos^{xe} RNA (Pickham *et al.*, 1992). Figure 2 shows data from one representative experiment that demonstrates that the $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot T^{161} \cdot \boldsymbol{A^{277}}$ and also the $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot \boldsymbol{A^{161}} \cdot \boldsymbol{D^{277}}$ RNAs induce $GVBD_{50}$ at approximately the same rate as the $\boldsymbol{A^{14}} \cdot \boldsymbol{F^{15}} \cdot T^{161} \cdot S^{277}$ mutant. However, the $T^{14} \cdot Y^{15} \cdot \boldsymbol{A^{161}} \cdot \boldsymbol{D^{277}}$ mutant, which retains the negative

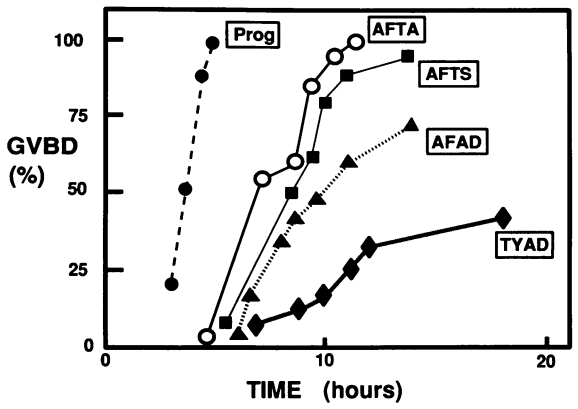

Figure 2. Time course of oocyte maturation. Oocytes were injected with wild-type or mutant *cdc2* RNAs and analyzed for GVBD by the appearance of a white spot on the animal pole. No spontaneous maturation was observed in unstimulated control oocytes incubated under identical conditions.

sites of regulatory phosphorylation Thr^{14} and Tyr^{15}, induced GVBD at an even slower rate. This result was not because of inefficient synthesis of the $T^{14} \cdot Y^{15} \cdot \boldsymbol{A^{161}} \cdot \boldsymbol{D^{277}}$ protein, as shown by efficient recovery of radiolabeled protein in Figure 1. It is possible that the low biological activity of the $T^{14} \cdot Y^{15} \cdot \boldsymbol{A^{161}} \cdot \boldsymbol{D^{277}}$ mutant reflects an indirect mechanism of induction of GVBD by this protein, as discussed below. Because of variations in different batches of oocytes, the exact time of $GVBD_{50}$ for any one mutant varied from experiment to experiment. However, the time required to reach $GVBD_{50}$ for any one mutant relative to the time required to reach $GVBD_{50}$ in progesterone-treated controls was consistent across experiments.

Importance of Ser^{277} for Regulation of cdc2 Kinase Activity

Each of the mutant proteins was also analyzed for in vitro kinase activity. In this experiment, oocytes were coinjected with RNAs encoding each of the mutant *cdc2*-TAG proteins, together with RNA encoding cyclin B1. In the resting oocyte, cyclin B proteins (B1 and B2) are present in limiting amounts, in contrast to excess amounts of endogenous *cdc2* protein (Kobayashi *et al.*, 1991); therefore, oocyte maturation can be readily induced by providing additional cyclin exogenously (Swenson *et al.*, 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989; Tachibana *et al.*, 1990; Freeman *et al.*, 1991). The coinjection of cyclin B1 RNA with each mutant *cdc2* RNA ensured that oocyte maturation would be induced in all oocytes, regardless of the cdc mutant being assayed. This strategy allowed recovery of the mutant *cdc2*-TAG proteins at $GVBD_{50}$ when endogenous MPF would be maximally activated. For each mutant, oocytes were lysed at $GVBD_{50}$, and one-half of the lysate was immunoprecipitated with anti-TAG antiserum (Figure 3B); as a control, the other half was immunoprecipitated with anti-*cdc2* antiserum (Figure 3A). As described previously, the anti-TAG antiserum recognizes only the *cdc2*-TAG proteins, and the anti-*cdc2* antiserum recognizes only the endogenous *cdc2* protein (Pickham *et al.*, 1992). The immunoprecipitates were then assayed for histone H1 kinase activity and analyzed by SDS-PAGE and autoradiography.

The results of this experiment are summarized in Table 2. When the mutant *cdc2* RNAs were coinjected with cyclin RNA, the oocytes reached $GVBD_{50}$ at ~4 to 6 h after injection, and the endogenous (wild-type) *cdc2* protein was clearly activated in all samples, as shown in Figure 3A. We previously used this approach to demonstrate H1 kinase activity associated with the $\boldsymbol{A^{14} \cdot F^{15}} \cdot T^{161} \cdot S^{277}$ mutant (Pickham *et al.*, 1992). Of the mutants assayed in Figure 3B, only the $\boldsymbol{A^{14} \cdot F^{15}} \cdot T^{161} \cdot \boldsymbol{A^{277}}$ mutant (lane 5) and wild-type *cdc2*-TAG (lane 4, labeled $T^{14} \cdot Y^{15} \cdot T^{161} \cdot S^{277}$) exhibited significant kinase activity in the anti-TAG immunoprecipitates. The $T^{14} \cdot Y^{15} \cdot T^{161} \cdot \boldsymbol{A^{277}}$ mutant also showed a low level of associated H1 kinase activity (lane 6), suggesting

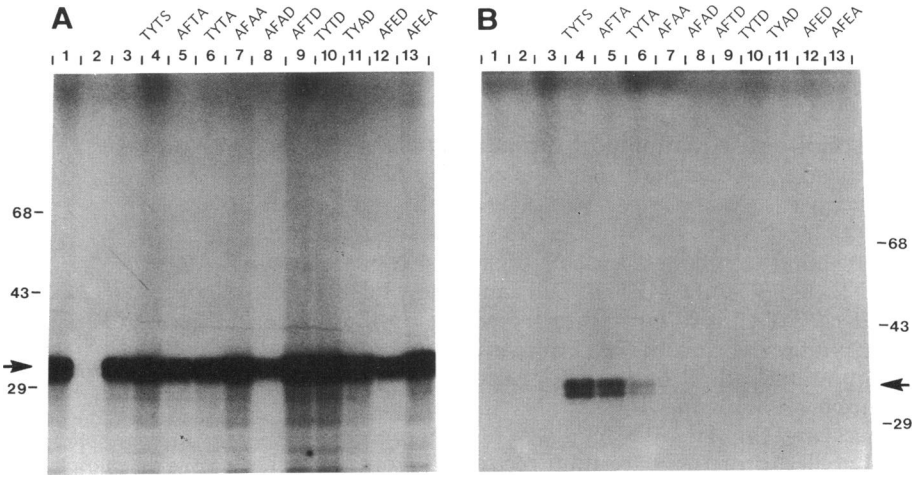

Figure 3. Histone H1 kinase activity of *cdc2* mutant proteins. Oocytes were coinjected with cyclin and *cdc2*-TAG RNAs. At $GVBD_{50}$ oocytes were lysed; half the lysate was immunoprecipitated with anti-*cdc2* antiserum, and half the lysate was immunoprecipitated with anti-TAG antiserum. After immunoprecipitation, in vitro histone H1 kinase assays were performed, and samples were analyzed by SDS-PAGE and autoradiography. A and B represent histone H1 kinase activities of the anti-*cdc2* immunoprecipitates and anti-TAG immunoprecipitates, respectively, from the same samples. Oocytes were treated as follows: lane 1, treated with progesterone; lane 2, untreated; lane 3, injected with water as a control; lanes 4–13, injected with in vitro–transcribed *cdc2*-TAG RNAs.

Table 2. Histone H1 kinase activity

cdc2 mutant	Histone H1 kinase activity[a]
$T^{14} \cdot Y^{15} \cdot T^{161} \cdot S^{277}$ [b]	++
$T^{14} \cdot Y^{15} \cdot T^{161} \cdot A^{277}$	+
$T^{14} \cdot Y^{15} \cdot T^{161} \cdot D^{277}$	−
$T^{14} \cdot Y^{15} \cdot A^{161} \cdot S^{277}$	−
$T^{14} \cdot Y^{15} \cdot A^{161} \cdot D^{277}$	−
$A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ [c]	++
$A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$	++
$A^{14} \cdot F^{15} \cdot T^{161} \cdot D^{277}$	−
$A^{14} \cdot F^{15} \cdot A^{161} \cdot S^{277}$ [c]	−
$A^{14} \cdot F^{15} \cdot A^{161} \cdot A^{277}$	−
$A^{14} \cdot F^{15} \cdot A^{161} \cdot D^{277}$	−
$A^{14} \cdot F^{15} \cdot E^{161} \cdot A^{277}$	−
$A^{14} \cdot F^{15} \cdot E^{161} \cdot D^{277}$	−

Histone H1 kinase activity of *cdc2* mutants. Associated histone H1 kinase activity was determined for cdc2 mutant proteins recovered from oocytes undergoing maturation induced by cyclin B1.

[a] Mutant $p34^{cdc2}$ was immunoprecipitated with anti-TAG antiserum, directed against the carboxy-terminal epitope tag, and then assayed for histone H1 kinase activity. In the second column, ++ indicates detectable kinase activity, + indicates a low level of kinase activity, and − indicates a lack of detectable histone H1 phosphorylation.

[b] Wild-type *cdc2* has the genotype $T^{14} \cdot Y^{15} \cdot T^{161} \cdot S^{277}$.

[c] Data for these mutants previously reported (Pickham *et al.*, 1992).

that it could be activated in oocytes undergoing maturation. The remaining *cdc2* mutant proteins displayed levels of H1 kinase activity that were near to background. Unexpectedly, the $T^{14} \cdot Y^{15} \cdot A^{161} \cdot D^{277}$ and $A^{14} \cdot F^{15} \cdot A^{161} \cdot D^{277}$ mutants (lanes 11 and 8, respectively), which can induce GVBD in the absence of coinjected cyclin, exhibited no detectable activity in this assay. This suggests that it is likely that some mutants can induce GVBD, not through their intrinsic kinase activity, but by indirectly activating endogenous *cdc2*/cyclin complexes. Alternatively, we cannot exclude the unlikely possibility that these mutants may possess intrinsic kinase activity that is undetectable when using histone H1 as an exogenous substrate in an in vitro kinase assay.

Analysis of Phosphopeptides in cdc2 Mutant Proteins

Phosphopeptide mapping of the mutant *cdc2* proteins was used to determine if Ser^{277} is actually phosphorylated on *cdc2* in oocytes. The $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ and $A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$ *cdc2*-TAG RNAs were injected into oocytes that were labeled with [^{32}P]orthophosphate (see MATERIALS AND METHODS), and the phosphorylated *cdc2*-TAG proteins were recovered by immunoprecipitation with anti-TAG antiserum. A typical autoradiogram of [^{32}P]-labeled *cdc2* proteins is shown in Figure 4, showing the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ mutant protein (lane 1) and the $A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$ mutant protein (lane 2). Labeled proteins were then recovered from the gel

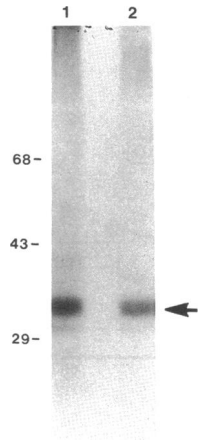

Figure 4. Immunoprecipitation and recovery of [^{32}P]-labeled *cdc2* mutant proteins. Oocytes expressing mutant *cdc2* proteins, after microinjection of in vitro–transcribed RNAs, were labeled with [^{32}P]orthophosphate as described in MATERIALS AND METHODS. Lane 1, $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ mutant protein; lane 2, $A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$ mutant protein.

and subjected to phosphoamino acid analysis and phosphopeptide mapping (see MATERIALS AND METHODS). Phosphoamino acid analysis showed that [^{32}P]-labeled $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ protein contained predominantly phosphoserine (Figure 5). Surprisingly, in several experiments, only trace amounts of phosphothreonine were detected despite the presence of Thr^{161} in this mutant protein. This suggests that only a small portion of the exogenously expressed *cdc2* mutant protein actually participates in the formation of active MPF and that the bulk of the exogenous *cdc2* protein may be biologically inert. This would not be surprising given that maturing oocytes contain a large excess of endogenous *cdc2*, compared with cyclin B proteins, and therefore only a small fraction of *cdc2* is assembled into active MPF complexes (Kobayashi *et al.*, 1991). When [^{32}P]-labeled $A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$ protein was also subjected to phosphoamino acid analysis, only phosphoserine was detected.

Figure 6 presents tryptic phosphopeptide maps for the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ (Figure 6A) and $A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$ (Figure 6B) mutant proteins. These maps revealed multiple phosphopeptides that appeared

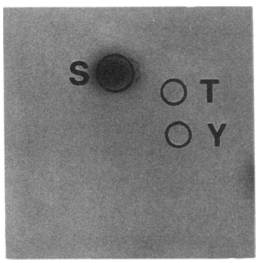

Figure 5. Phosphoamino acid analysis of the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ mutant *cdc2* protein. The [^{32}P]-labeled *cdc2* protein was transferred to nitrocellulose, digested with trypsin, and then subsequently treated with 6 N HCl at 110°C and separated by electrophoresis in two dimensions in the presence of cold phosphoamino acids. The position of unlabeled phosphoamino acid markers is indicated for phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y).

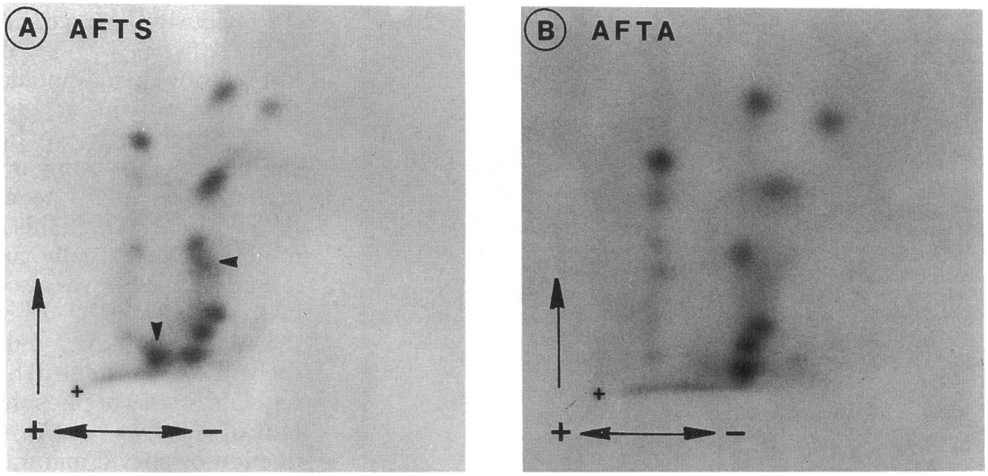

Figure 6. Tryptic phosphopeptide maps of *cdc2*-TAG mutants. The [^{32}P]-labeled *cdc2* proteins shown in Figure 4 were transferred to nitrocellulose, digested with trypsin, and then spotted on chromatography plates. Samples were separated by pH 1.9 electrophoresis followed by ascending chromatography in phosphochromatography buffer. A: $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ mutant protein. B: $A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$ mutant *cdc2* protein.

to be identical, which was confirmed by a mixing experiment. However, two phosphopeptides present in the tryptic map of the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ protein (see arrows, Figure 6A) appeared to be missing in the $A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$ mutant. However, the peptide containing Ser^{277}, $IS^{277}AR$, is predicted to be in the lower right side of the peptide map because of its small size and net positive charge at pH 1.9, together with its hydrophilicity. Thus, it seemed unlikely that either of the unique peptides in the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ tryptic phosphopeptide map would contain Ser^{277}.

To confirm this, two different peptides were synthesized, one corresponding to the predicted tryptic phosphopeptide containing Ser^{277}, $IS^{277}AR$. The second peptide (referred to as #794), YDPAKR$IS^{277}AR$KALL, contains this sequence and the surrounding region to determine if the correct trypsin cleavage sites had been identified. The second peptide, if cleaved as predicted, would yield ISAR; however, as tryptic cleavage after Arg^{275} could be inhibited by phosphorylation of Ser^{277} (Boyle *et al.*, 1991), another possible cleavage product of this peptide would be RISAR. Both peptides were enzymatically phosphorylated, as described in MATERIALS AND METHODS, after which the phosphorylated peptides were purified by two dimensional electrophoresis and chromatography on thin-layer chromatography plates and eluted. The phosphorylated 794 peptide was additionally digested with trypsin, using identical conditions as for trypsin digestion of *cdc2* proteins, and the resulting phosphopeptide was repurified by two-dimensional electrophoresis and chromatography. Each of the synthetic peptides, one derived by phosphorylation of $IS^{277}AR$ and the other by phosphorylation of YDPAKR$IS^{277}AR$KALL followed by trypsin digestion, was analyzed in comparison with the tryptic phosphopeptide map of the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ protein. Neither of the synthetic phosphopeptides comigrated with any phosphopeptide present in the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ map. These results indicate that Ser^{277} does not represent a major site of phosphorylation during oocyte maturation up to the time of GVBD. As discussed previously, however, because *cdc2* is quantitatively in excess compared with cyclin B proteins, only a small proportion of the total exogenous *cdc2* may be assembled into active complexes. Thus, we cannot exclude the possibility that Ser^{277} may be phosphorylated on a subset of the exogenous *cdc2* molecules and that this population may in fact be responsible for the biological activity exhibited by the *cdc2* mutant.

These experiments leave unresolved the identity of the two phosphopeptides present in the map of the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ *cdc2* protein (Figure 6A), which are absent in the map of the $A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$ protein (Figure 6B). We would speculate that these differences in phosphorylation sites might reflect subtle conformational differences between the two proteins. In addition, we have no data concerning the identification of the other seven major phosphopeptides in the map of the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ protein. With a total of 20 different Ser residues present in the *cdc2* protein, however, the identification of these phosphorylation sites will require considerable future effort.

DISCUSSION

To analyze the importance of Ser^{277} of *cdc2* in *Xenopus* oocytes, we created and analyzed a series of Ser^{277} mutants (see Table 1). The mutation of Ser^{277} → Ala^{277} had no apparent effect upon *cdc2* activity, as demonstrated by the similar phenotypes of the $T^{14} \cdot Y^{15} \cdot T^{161} \cdot A^{277}$ single mutant compared with the wild-type $T^{14} \cdot Y^{15} \cdot T^{161} \cdot S^{277}$ and as demonstrated by the triple mutant $A^{14} \cdot F^{15} \cdot T^{161} \cdot A^{277}$ compared with the double mutant $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$. However, the mutation of Ser^{277} → Asp^{277} while having no apparent biological effect on its own, was inhibitory when placed in the triple mutant $A^{14} \cdot F^{15} \cdot T^{161} \cdot D^{277}$.

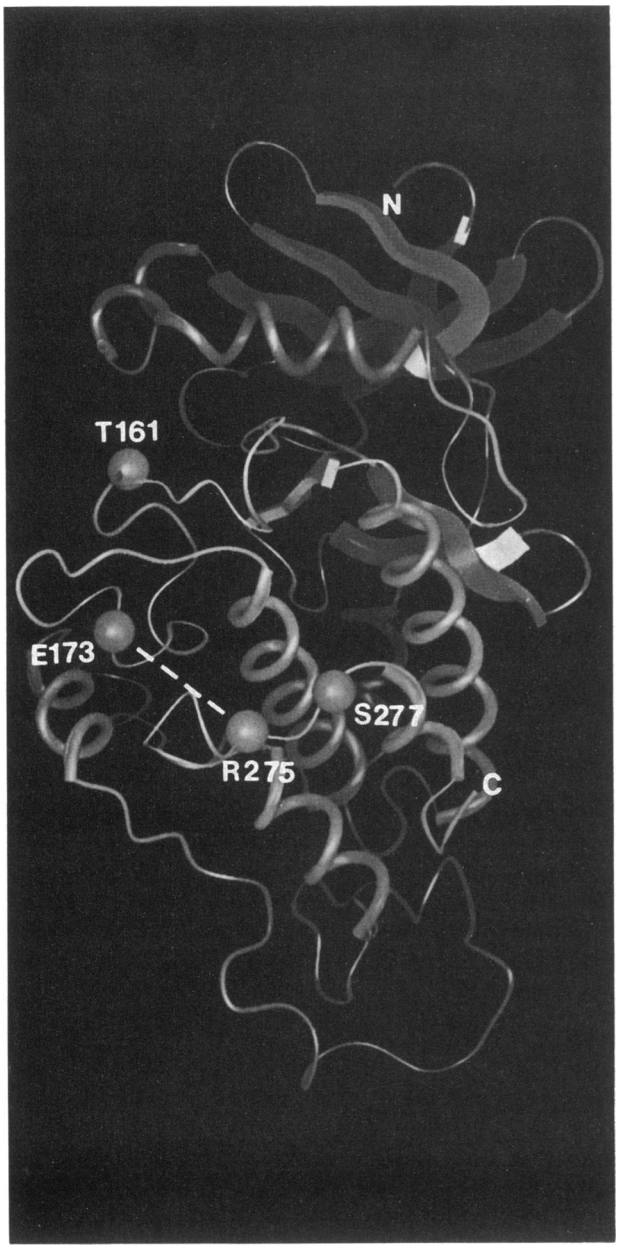

Figure 7. Location of Ser^{277} in predicted structure of *cdc2* protein kinase. The upper lobe of the molecule consists largely of β strands and contains the nucleotide binding domain, including the key regulatory phosphorylation sites Thr^{14} and Tyr^{15} (not shown), whereas the lower lobe contains more α-helical structure and constitutes the catalytic domain (Marcote *et al.*, 1993). The α-carbon atoms of Thr^{161}, Glu^{173}, Arg^{275}, and Ser^{277} are indicated by the CPK balls, as labeled. The N and C termini of the *cdc2* protein are indicated as well. In the molecular model of *cdc2*, residues Arg^{275} and Glu^{173} are predicted to form a buried salt bridge (– – –) (Marcote *et al.*, 1993). Note that although the *Schizosaccharomyces pombe cdc2* sequence was used to create the molecular model shown here (Marcote *et al.*, 1993), the *Xenopus cdc2* would be almost superimposable because of the high degree of sequence relatedness (87% identity) between the two primary amino acid sequences. Phosphorylation at Ser^{277} may sufficiently perturb this salt bridge so as to affect the loop containing Glu^{173}, which also contains Thr^{161}.

Previously, we have shown that the biological activity of the $A^{14} \cdot F^{15} \cdot T^{161} \cdot S^{277}$ double mutant was completely lost by subsequent mutation of $Thr^{161} \rightarrow Ala^{161}$ to create the triple mutant $A^{14} \cdot F^{15} \cdot A^{161} \cdot S^{277}$ (Pickham *et al.*, 1992). Surprisingly, we show here that biological activity can be restored to this mutant by additional mutation of $Ser^{277} \rightarrow Asp^{277}$ to create the quadruple mutant $A^{14} \cdot F^{15} \cdot A^{161} \cdot D^{277}$. This result suggests that the Asp^{277} mutation can partially compensate for the Ala^{161} mutation. Moreover, we also observed a similar activation of the $T^{14} \cdot Y^{15} \cdot A^{161} \cdot D^{277}$ mutant, which incorporates the Ala^{161} and Asp^{277} mutations in a background with the wild-type residues Thr^{14} and Tyr^{15} that are negative regulatory sites of phosphorylation. This is the first mutant of *Xenopus cdc2* in which we have observed induction of oocyte maturation by a mutant that retains the negative regulatory sites Thr^{14} and Tyr^{15}.

The biological activity of the mutants described here, in the absence of peptide mapping data, might readily be interpreted to suggest that Ser^{277} may be a phosphorylation site on the *Xenopus cdc2* protein during oocyte maturation. However, although there are clearly multiple sites of serine phosphorylation on *cdc2* during oocyte maturation (see Figure 6 and Gabrielli *et al.*, 1992), biochemical analysis of [^{32}P]-labeled *cdc2* protein recovered from maturing oocytes indicates that Ser^{277} is apparently not a major site of serine phosphorylation on *cdc2* during *Xenopus* oocyte maturation. In contrast, in cycling chicken cells *cdc2* is phosphorylated on Ser^{277}, peaking during G1 (Krek and Nigg, 1991a), although the importance of this phosphorylation is unknown. We cannot rule out the possibility, however, that Ser^{277} may be phosphorylated on a subset of *cdc2* molecules that are biologically active.

In the experiments we describe here, although we were unable to detect Ser^{277} phosphorylation, mutation of $Ser^{277} \rightarrow Asp^{277}$ may mimic a mitotic control mechanism—one that does not normally exist in oocytes. Regardless of the mechanism, our results clearly demonstrate a potential regulatory role for Ser^{277} in *Xenopus cdc2*.

Significantly, our results describe for the first time a mutant *cdc2* that can induce GVBD in the absence of a phosphorylatable residue at position 161. It has been demonstrated that phosphorylation of Thr^{161} is required for *cdc2* activity and that its dephosphorylation is required for normal cell cycle regulation (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Lorca *et al.*, 1992; Solomon *et al.*, 1992). In our previous work, we showed that the presence of an Ala or Glu at position 161 blocks the ability of a Ala^{14}/Phe^{15} mutant to induce GVBD in *Xenopus* oocytes (Pickham *et al.*, 1992). However, here we have shown that two independent *cdc2* constructs that include the Ala^{161} mutation can induce GVBD in the presence of a secondary mutation, namely Asp^{277}. Both of the Ala^{161} *cdc2* mutants show a lack of histone H1 kinase activity as would be expected (Ducommun *et al.*,

1991; Gould *et al.*, 1991; Pickham *et al.*, 1992; Solomon *et al.*, 1992). This suggests that there may be another mechanism, other than formation of an active MPF complex, by which they can induce GVBD, although the nature of this indirect mechanism remains unclear at the present time. However, several possible options can be proposed. One is that these mutants may titrate out an inhibitory factor, such as the Thr^{14}/Tyr^{15}-kinase Wee1 (Featherstone *et al.*, 1991; Parker *et al.*, 1991, 1992). Another possibility is that histone H1 phosphorylation may not be a reliable indicator of MPF activity. A third is that the mutants may actually exhibit an undetectably low but catalytic level of kinase activity.

Further information can be learned from the structure of *cdc2* derived by molecular modeling, based on the crystallographic structure of the cAMP dependent protein kinase (Marcote *et al.*, 1993). As shown schematically in Figure 7, one can see that Ser^{277} is not near the ATP-binding site, nor is it near the cyclin binding site. However, Ser^{277} does lie near Arg^{275}, which participates in a buried salt bridge with Glu^{173} and which is located in the same loop that also contains the regulatory phosphorylation site Thr^{161}. This salt bridge corresponds to the salt bridge between Arg^{280} and Glu^{208} in the native cAMP dependent protein kinase structure (Knighton *et al.*, 1991), and these residues are conserved throughout the family of cyclin dependent protein kinases (De Bondt *et al.*, 1993). Phosphorylation at Ser^{277} or, alternatively, mutation of Ser^{277} to an acidic residue, might be expected to perturb or disrupt this salt bridge in *cdc2*, leading to conformational effects involving Thr^{161}. Indeed, these *intramolecular* effects on *cdc2* conformation would be expected to exert effects on *intermolecular* interactions between *cdc2* and other regulatory or binding proteins. Thus, mutations affecting Ser^{277} might alter the normal regulation of *cdc2* activity as a result of altered interactions with regulatory proteins whose interactions with *cdc2* are determined in part by Thr^{161} and whether it is phosphorylated. We would also predict that other mutations in the vicinity of Arg^{275} might similarly perturb its ability to form an ion pair with Glu^{173}, leading to long-range effects on Thr^{161} and thereby altering the regulation of *cdc2* activity.

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