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Cell Biology

Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation

(cytoplasmic retention signal/nuclear translocation)

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ABSTRACT **M-phase promoting factor or maturation promoting factor, a key regulator of the $G_2 \rightarrow M$ transition of the cell cycle, is a complex of cdc2 and a B-type cyclin. We have previously shown that *Xenopus* cyclin B1 has five sites of Ser phosphorylation, four of which map to a recently identified cytoplasmic retention signal (CRS). The CRS appears to be responsible for the cytoplasmic localization of B-type cyclins, although the underlying mechanism is still unclear. Phosphorylation of cyclin B1 is not required for cdc2 binding or cdc2 kinase activity. However, when all of the Ser phosphorylation sites in the CRS are mutated to Ala to abolish phosphorylation, the mutant cyclin $B1^{Ala}$ is inactivated; activity can be enhanced by mutation of these residues to Glu to mimic phosphoserine, suggesting that phosphorylation of cyclin B1 is required for its biological activity. Here we show that biological activity can be restored to cyclin $B1^{Ala}$ by appending either a nuclear localization signal (NLS), or a second CRS domain with the Ser phosphorylation sites mutated to Glu, while fusion of a second CRS domain with the Ser phosphorylation sites mutated to Ala inactivates wild-type cyclin B1. Nuclear histone H1 kinase activity was detected in association with cyclin $B1^{Ala}$ targeted to the nucleus by a wild-type NLS, but not by a mutant NLS. These results demonstrate that nuclear translocation mediates the biological activity of cyclin B1 and suggest that phosphorylation within the CRS domain of cyclin B1 plays a regulatory role in this process. Furthermore, given the similar *in vitro* substrate specificity of cyclin-dependent kinases, this investigation provides direct evidence for the hypothesis that the control of subcellular localization of cyclins plays a key role in regulating the biological activity of cyclin-dependent kinase–cyclin complexes.**

M-phase promoting factor or maturation promoting factor (MPF), the key regulator of the $G_2 \rightarrow M$ transition during the cell cycle (1, 2), is regulated by phosphorylation of both of its component proteins: the serine/threonine protein kinase cdc2 and a B-type cyclin. Three phosphorylation sites govern the activity of cdc2: phosphorylation of Thr-14 and Tyr-15 inhibits cdc2 activity, while phosphorylation of Thr-161 acts as a positive regulatory signal (3–9).

B-type cyclins are phosphorylated in a variety of cell types, including somatic cells, oocytes, and embryos (10–12). Phosphorylation of B-type cyclins temporally correlates with MPF activation (11, 12). Five phosphorylation sites have been identified for *Xenopus* cyclin B1 at Ser-2, -94, -96, -101, and -113 (13, 14). Phosphorylation of cyclin B1 is required for its biological activity, as demonstrated by the fact that mutation of these five Ser phosphorylation sites to Ala inactivates cyclin B1, whereas mutation of the same residues to Glu to mimic phosphoserine enhances the activity of cyclin B1 (13). However, the precise role of phosphorylation in regulating cyclin activity has remained obscure. It is known that phosphorylation of cyclin B1 is not required for cdc2 kinase activity or cdc2 binding (13). In *Xenopus* oocytes, phosphorylation of cyclin B1 does not affect its stability before meiosis or its destruction between meiosis I and II or after egg fertilization (13).

In human cyclin B1, a cytoplasmic retention signal (CRS) has been identified that is highly conserved among B-type cyclins in higher eukaryotes. The CRS appears to retain cyclin B1 within the cytoplasm during G_2, although the underlying mechanism is unknown (15). Interestingly, four of the five phosphorylation sites in *Xenopus* cyclin B1, Ser-94, Ser-96, Ser-101, and Ser-113, are located within this CRS domain and are also conserved among B-type cyclins in higher eukaryotes (13, 15). B-type cyclins have been observed to translocate from the cytoplasm to the nucleus at the beginning of M phase in both cultured animal cells and starfish oocytes (16–18), although the biological role and the regulation of this nuclear localization is undetermined.

Because phosphorylation occurs at sites within the CRS and both phosphorylation and nuclear translocation of cyclin B1 happen at M phase, we wished to examine whether phosphorylation controls the activity of cyclin B1 by regulating its subcellular localization. We propose that the nuclear localization of cyclin B1 is regulated by phosphorylation at sites within the CRS domain, which abolishes cytoplasmic retention and allows nuclear translocation.

MATERIALS AND METHODS

Oocyte Microinjection. Oocyte microinjection was performed as described (13). For each construct, a minimum of 20 stage VI oocytes were injected, and three independent experiments were performed. *Xenopus* oocyte maturation, induced by microinjection of *in vitro* synthesized RNAs encoding cyclin B1 fusion proteins, was scored as the percentage of microinjected stage VI oocytes that underwent germinal vesicle breakdown (%GVBD). Isolation of nuclei from oocytes was performed as described (19). Briefly, defolliculated oocytes were torn in the middle of the animal pole in "intracellular medium" (102 mM KCl/11.1 mM NaCl/7.2 mM K_2HPO_4/4.8 mM KH_2PO_4, pH 7.0/2% BSA). The nuclei were squeezed out of the oocytes and washed in the same medium. Then the nuclei and the enucleated oocytes were transferred to lysis buffer and subjected to immunoprecipitation and histone H1 kinase assay, as described (13). To detect cdc2 binding, samples of oocyte lysates expressing cyclin B1 fusion proteins were im-

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Abbreviations: CRS, cytoplasmic retention signal; NLS, nuclear localization signal; MPF, maturation promoting factor or M phase promoting factor; GVBD, germinal vesicle breakdown.

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munoprecipitated with the mAb P5D4 directed against the epitope tag, subjected to 12.5% SDS/PAGE, and then immunoblotted. A mouse mAb specific to p34*cdc2* (Santa Cruz Biotechnology) was used as the primary antibody, and peroxidase-labeled anti-mouse Ig (Amersham) served as the secondary antibody; cdc2 proteins were then detected by ECL (Amersham).

Construction of Cyclin B1-Derived Fusion Proteins. A nuclear localization signal (NLS) or mutant NLS (NLS^{mut}) was fused to the N terminus of derivatives of *Xenopus* cyclin B1. The NLS is a 16-amino acid peptide with a bipartite structure derived from *Xenopus* nucleoplasmin (20). In the NLS^{mut}, the six basic amino acids in the bipartite signal were mutated to Asn to inactivate the NLS (20). B1 refers to wild-type cyclin B1. $B1^{Ala}$ has Ser → Ala substitutions at residues 94, 96, 101, and 113 to abolish phosphorylation of cyclin B1, and $B1^{Glu}$ has Ser → Glu substitutions at residues 94, 96, 101, and 113 to mimic phosphoserine (13). The CRS used in N-terminal fusions consists of residues 78–127 of *Xenopus* cyclin B1 (15). CRS^{Ala} and CRS^{Glu} contain Ser → Ala or Ser → Glu substitutions, respectively, at residues 94, 96, 101, and 113. N-terminal fusions of the NLS or CRS had the first 28 or 34 amino acids of cyclin B1 deleted, respectively, which has no effect on its biological activity (21). All constructs used in this work were epitope-tagged at the C terminus with an epitope for the mouse mAb P5D4 (22). C-terminal epitope-tagging of cyclin B1 has no effect on its biological activity (13). NLS–B1, NLS–$B1^{Glu}$, NLS^{mut}–B1, and NLS^{mut}–$B1^{Glu}$ were made by ligating pairs of complementary oligonucleotides encoding either NLS or NLS^{mut} into *Eco*RI and *Sty*I sites of cyclin B1 or cyclin $B1^{Glu}$ (13, 20, 23) pSP64(polyA) vector. NLS–$B1^{Ala}$ was also made with oligonucleotides encoding the NLS but ligated into *Eco*RI and *Bgl*I sites of cyclin $B1^{Ala}$ (13) in a pSP64(polyA) vector. NLS^{mut}–$B1^{Ala}$ was created by exchanging *Bgl*I fragments of NLS^{mut}–B1 and cyclin $B1^{Ala}$ (13). Fusion constructs containing CRS^{Ala} or CRS^{Glu} used similar cloning strategies. The design of the N-terminal NLS and CRS fusion proteins included a Kozak sequence for optimal translational initiation (24). All sequences derived from synthetic oligonucleotides were confirmed by DNA sequencing.

Immunofluorescence. COS-1 cells on coverslips were transiently transfected with cyclin B1 mutants in the vector pCDNA3 under cytomegalovirus promoter control. Two days after transfection, the cells were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100, 0.2 M glycine, and 2.5% fetal bovine serum in PBS. Triple label immunofluorescence was performed. Primary antibody mAb P5D4 (22) was used to locate the epitope-tagged cyclin B1 derivatives and a fluorescein-conjugated goat anti-mouse antiserum as the secondary antiserum. Antilamin A, B, C primary antiserum was used to detect the nuclear membrane and visualized with a rhodamine-conjugated goat anti-rabbit secondary antibody. Finally, DNA in the nuclei of cells was detected with Hoechst dye 33342.

RESULTS

NLS Activates Cyclin $B1^{Ala}$. To examine the importance of nuclear translocation for cyclin B1 function and the role of phosphorylation within the CRS in regulating this nuclear translocation, chimeric proteins were designed that contain a NLS or a second CRS domain appended to the N terminus of cyclin B1, as diagrammed in Fig. 1. Expression of all constructs was verified by immunoprecipitation and SDS/PAGE analysis from radiolabeled lysates of microinjected oocytes, which yielded proteins of the expected molecular weight as shown in Fig. 2*A*.

The biological activity of these constructs, as indicated by meiotic maturation of *Xenopus* oocytes, was assayed by microinjection of resting stage VI *Xenopus* oocytes with *in vitro*-synthesized RNAs encoding the various fusion proteins. The total percentage of oocytes that reached GVBD during overnight incubation is presented in Fig. 1. We first characterized chimeric proteins consisting of wild-type or mutant forms of a well characterized NLS, derived from *Xenopus* nucleoplasmin (20), fused with wild-type or mutant forms of cyclin B1. Fig. 3 presents the kinetics of oocyte meiotic maturation in response to expression of these chimeric proteins. As shown previously (13), cyclin $B1^{Ala}$ is unable to induce oocyte maturation (Figs. 1 and 3*A*). Fusion of the NLS to cyclin $B1^{Ala}$ completely restored its activity; in contrast, fusion of the nonfunctional NLS mutant did not restore the biological activity as shown by the failure of NLS^{mut}–$B1^{Ala}$ to induce oocyte maturation (Figs. 1 and 3*A*). These results suggest that nuclear translocation of cyclin B1 may mediate its biological activity, and also suggest that elimination of phosphorylation abolishes nuclear translocation. In support of this, B-type cyclins have been observed to undergo nuclear translocation at the beginning of M phase in cultured animal cells and starfish oocytes (16–18).

To ensure that the differences in biological activity did not result from varying protein levels, we compared the expression of the fusion proteins in oocytes at 5 h after injection, prior to the time when maturing oocytes begin to undergo GVBD. As shown in Fig. 2*A*, no significant differences were observed

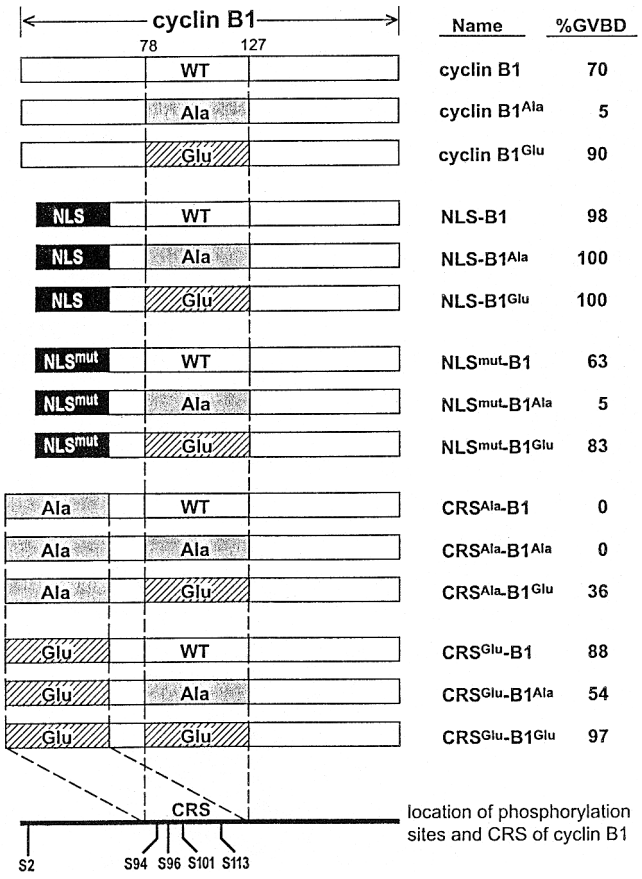

FIG. 1. Structure and activity of cyclin B1-derived fusion proteins. A NLS or nonfunctional mutant NLS (NLS^{mut}) was fused to the N terminus of derivatives of *Xenopus* cyclin B1. B1 refers to wild-type cyclin B1. $B1^{Ala}$ has Ser → Ala substitutions at residues 94, 96, 101, and 113, and $B1^{Glu}$ has Ser → Glu substitutions at residues 94, 96, 101, and 113. CRS^{Ala} and CRS^{Glu} contain Ser → Ala or Ser → Glu substitutions, respectively, at residues 94, 96, 101, and 113. *Xenopus* oocyte maturation, induced by microinjection of *in vitro* synthesized RNAs encoding these proteins, was scored as the percentage of microinjected stage VI oocytes that underwent GVBD. The percentage reaching GVBD represents the mean of three independent experiments.

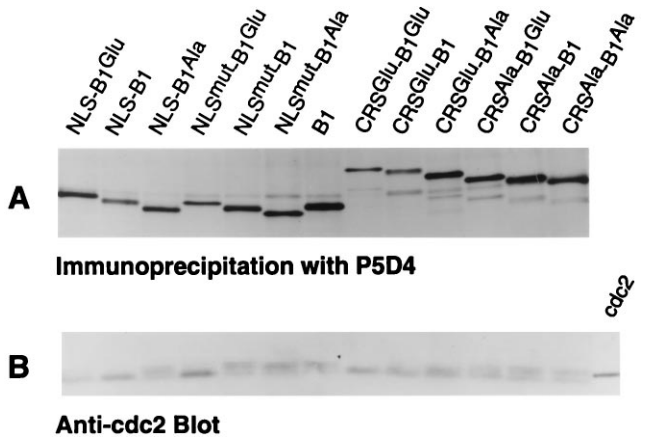

FIG. 2. Expression and cdc2 binding of cyclin B1 fusion proteins. Microinjected oocytes were labeled for 5 h as described (13) using 0.5 mCi/ml (1 Ci = 37 GBq) [^{35}S]Met and 0.25 mCi/ml [^{35}S]Cys. (*A*) Oocytes were lysed and half of the lysate was subjected to immunoprecipitation to recover labeled cyclin proteins using a mAb directed against the epitope tag. Proteins were analyzed by 12.5% SDS/PAGE and detected by fluorography. (*B*) The other half of each sample was immunoprecipitated as in *A*, and cdc2 proteins were detected by immunoblotting as described. The last lane in *B* shows *in vitro* translated cdc2 as a control.

between the biologically active and inactive cyclin B1 fusion proteins with respect to their level of protein expression. In a separate experiment (data not shown), we also examined the expression of the inactive mutant proteins 10 h after injection, and found that the relative amounts of protein had increased compared with the 5-h time point. These data indicate that the lack of biological activity of NLS^{mut}–B1^{Ala} was not the result of decreased expression.

To confirm that NLS–B1^{Ala} is directed to the nucleus and is complexed with cdc2 as active MPF, we examined the histone H1 kinase activity associated with NLS–B1^{Ala} and NLS^{mut}–B1^{Ala} from isolated nuclei versus enucleated oocytes, at 2.5 and 4.5 h after injection. As presented in Fig. 4, histone H1 kinase activity was readily detected in association with NLS–B1^{Ala}, recovered by immunoprecipitation from nuclear lysates (lanes 6 and 12). In contrast, only background levels of associated H1 kinase activity were detected in the nuclear samples of oocytes expressing the biologically inactive mutant, NLS^{mut}–B1^{Ala} (lanes 5 and 11). These differences in the associated *in vitro* H1 kinase activity were not due to an inability of the inactive mutant, NLS^{mut}–B1^{Ala}, to bind cdc2. This is demonstrated in Fig. 2*B*, which shows that all of the cyclin B1 fusion proteins examined here are able to bind cdc2, which is detected as three closely migrating species differing in their phosphorylation states (9). These data indicate that only NLS–B1^{Ala}, but not NLS^{mut}–B1^{Ala}, accumulates in an active form within the nuclei of oocytes as they progress toward GVBD. Only basal levels of histone H1 kinase activity were found in the cytoplasmic fraction of oocytes expressing NLS–B1^{Ala} and NLS^{mut}–B1^{Ala}.

As shown in Fig. 3*B*, fusion of the NLS to cyclin B1 or cyclin B1^{Glu} significantly accelerated oocyte maturation, as compared with fusions containing the mutant NLS. This also was not due to significant differences in levels of expression of the cyclin B1 fusion proteins or in their ability to bind cdc2, as shown in Fig. 2. These data are consistent with a role for nuclear translocation of cyclin B1 in promoting the $G_2 \rightarrow M$ transition of the cell cycle.

CRS^{Ala} Inactivates Cyclin B1. A recently identified CRS (15) contains four out of five phosphorylation sites in cyclin B1. To examine the role of phosphorylation in regulation of CRS activity, we fused a mutant CRS domain, CRS^{Ala}, containing Ser → Ala substitutions to abolish phosphorylation sites, to the N terminus of wild-type cyclin B1 (Fig. 1).

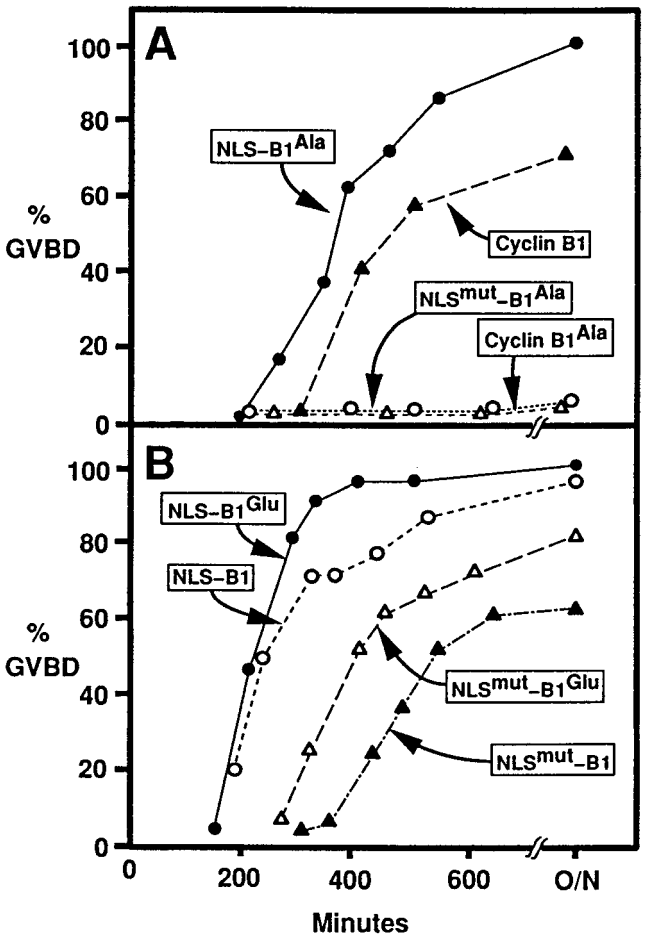

FIG. 3. Kinetics of oocyte maturation in response to cyclin B1 fusion proteins containing a wild-type NLS or NLS^{mut} derived from nucleoplasmin. *In vitro*-synthesized RNA of each construct was microinjected into a minimum of 20 stage VI oocytes. Oocytes were monitored every 30 min to determine whether they had reached GVBD. (*A*) NLS–B1^{Ala}, NLS^{mut}–B1^{Ala}, and cyclin B1^{Ala}. (*B*) NLS–B1, NLS–B1^{Glu}, NLS^{mut}–B1, and NLS^{mut}–B1^{Glu}.

Microinjection of *in vitro* synthesized RNA encoding CRS^{Ala}–B1 into *Xenopus* oocytes demonstrated that the CRS^{Ala} completely abolished the biological activity of cyclin B1 in this fusion protein (Figs. 1 and 5*A*). In contrast to CRS^{Ala}, the N-terminal fusion of CRS^{Glu}, containing Ser → Glu substitutions at the phosphorylation sites to mimic phosphoserine, did not significantly affect the biological activity of cyclin B1 (Figs. 1 and 5*A*). As shown in Fig. 2, the level of protein expression and the ability to bind cdc2 are comparable for the different fusion proteins. For the mutants that did not induce GVBD, such as CRS^{Ala}–B1, the level of protein expression was also examined at 10 h after injection (data not shown) and was found to have increased compared with the level of expression observed at 5 h (Fig. 2). Thus, the inability of some mutants to induce GVBD was not due to decreased expression or inability to bind to cdc2. These results suggest that CRS^{Ala} provides for constitutive cytoplasmic retention when fused to wild-type cyclin B1.

Ser → Glu Mutations Compared with Ser → Ala Mutations. We also investigated the effect of fusing CRS^{Glu} to the inactive cyclin B1^{Ala}. As shown in Figs. 1 and 5*B*, CRS^{Glu}–B1^{Ala} exhibited partial biological activity. Similarly, the construct CRS^{Ala}–B1^{Glu} also exhibited partial biological activity. Both of these fusion proteins have two CRS domains, one containing Ser → Glu substitutions and the other containing Ser → Ala substitutions at the phosphorylation sites; these should be

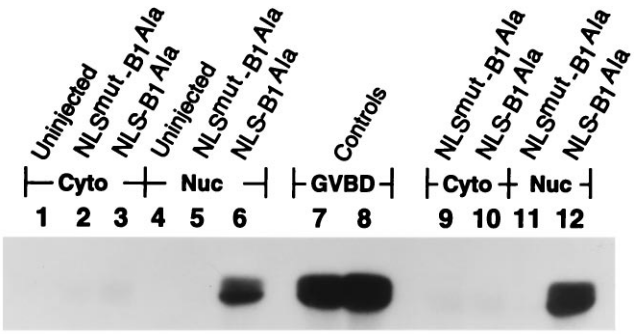

FIG. 4. Localization of NLS–B1^{Ala} and NLS^{mut}–B1^{Ala} associated H1 kinase activity. Nuclei were manually isolated at either 2.5 or 4.5 h from oocytes microinjected with *in vitro*-synthesized RNA encoding NLS–B1^{Ala} and NLS^{mut}–B1^{Ala}. Nuclear and cytoplasmic fractions were then immunoprecipitated using P5D4 antibody to recover the epitope-tagged cyclin B1 fusion proteins, and immunoprecipitates were examined for histone H1 kinase activity, associated with active MPF. Phosphorylated histone H1 was detected by autoradiography after separation by 15% SDS/PAGE. Samples from nuclei (Nuc) or from enucleated oocytes (Cyto) were analyzed at two different time points after microinjection; 2.5 h (lanes 2, 3, 5, and 6) and 4.5 h (lanes 9–12). As a negative control, the cytoplasmic (lane 1) and nuclear (lane 4) fractions from uninjected oocytes were similarly analyzed. For lanes 1–6 and 9–12, each sample corresponds to three nuclei or enucleated oocytes. As a positive control, oocytes that reached GVBD (at ≈5.5 h) after microinjection with RNA encoding NLS–B1^{Ala} were similarly analyzed for histone H1 kinase activity. Lanes 7 and 8 correspond to one-quarter and to one-half of an oocyte, respectively.

compared with the inactive control, CRS^{Ala}–B1^{Ala}, and the active control CRS^{Glu}–B1^{Glu}, each of which has two identical CRS domains (Figs. 1 and 5*B*).

These results demonstrate that CRS^{Glu}, mimicking a phosphorylated CRS domain, can rescue the biological activity of an inactive cyclin B1 derivative whether appended at the N terminus or at its usual location within cyclin. The intermediate levels of activity exhibited by CRS^{Glu}–B1^{Ala} and by CRS^{Ala}–B1^{Glu} also suggest that neither of the fully substituted CRS domains, CRS^{Glu} or CRS^{Ala}, can act in a completely dominant fashion over the other; rather, they appear to act as codominant localization signals.

To directly examine nuclear translocation, triple label indirect immunofluorescence was performed on COS-1 cells transiently transfected with the cyclin B1 mutants. All of the constructs contained an epitope tag derived from vesicular stomatitis virus glycoprotein that was recognized by the murine mAb P5D4 (22) (Fig. 6 *Right*). As seen in Fig. 6 *B*, *C*, and *F*, the constructs that exhibited biological activity, cyclin B1^{Glu}, NLS–B1^{Ala}, and CRS^{Glu}–B1^{Glu}, also exhibited nuclear localization. The antilamin staining confirmed that the nuclear membrane appeared to be intact (Fig. 6 *Center*). The cytoplasmic staining (Fig. 6 *A*, *D*, and *E*) exhibited by cyclin B1^{Ala}, NLS^{mut}–B1^{Ala}, and CRS^{Ala}–B1^{Ala}, was also consistent with their inability to induce oocyte maturation.

Phosphorylation at Ser-2 Is Unimportant for Biological Activity. Ser-2 in *Xenopus* cyclin B1 is not conserved among B-type cyclins (16, 23), and resides outside the CRS domain (13, 15). However, because Ser-2 is removed in the cyclin B1 fusion proteins examined here, it was important to exclude the possibility that this phosphorylation site plays a regulatory role in the induction of *Xenopus* oocyte maturation by cyclin B1. To examine this, we made the following cyclin B1 mutants: S2A-QDE with Ser 2 mutated to Ala and Ser-94, Ser-96, Ser-101, and Ser-113 mutated to Glu, and S2A-QDA with Ser-2 mutated to Glu and Ser-94, Ser-96, Ser-101, and Ser-113 mutated to Ala. These two mutants were assayed in oocytes along with wild-type cyclin B1, cyclin B1^{Ala} (all five Ser phosphorylation sites mutated to Ala), and cyclin B1^{Glu} (all

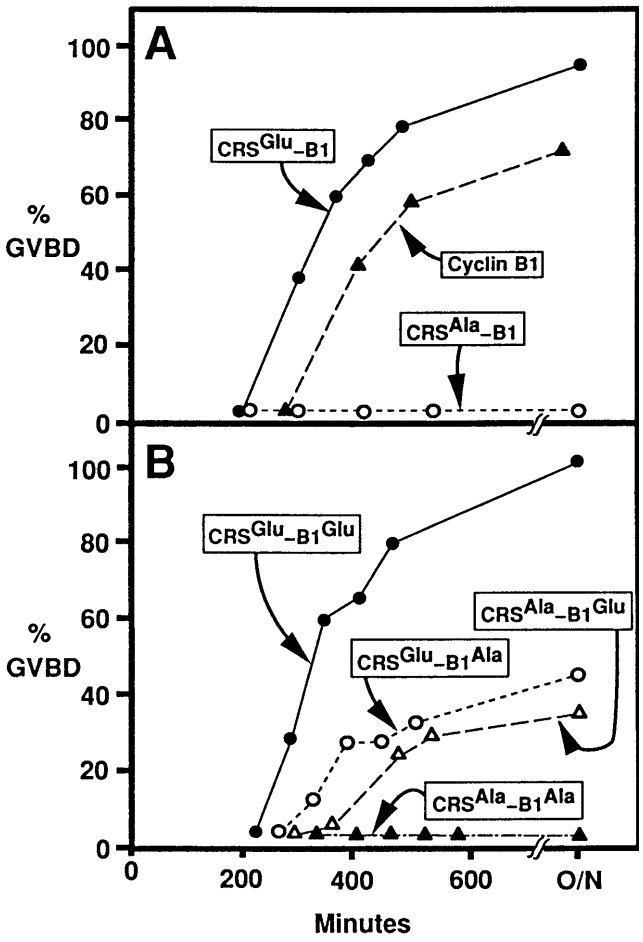

FIG. 5. Kinetics of oocyte maturation in response to cyclin B1 fusion proteins containing a second CRS appended at the N terminus. Procedures for RNA injections and monitoring oocytes were the same as in Fig. 3. (*A*) CRS^{Ala}–B1 and CRS^{Glu}–B1. (*B*) CRS^{Ala}–B1^{Ala}, CRS^{Ala}–B1^{Glu}, CRS^{Glu}–B1^{Ala}, and CRS^{Glu}–B1^{Glu}.

five Ser phosphorylation sites mutated to Glu). Fig. 7 shows oocyte maturation kinetics induced by cyclin B1 and its mutants. The activity of cyclin B1^{Glu} and S2A-QDE was similar and was enhanced as compared with wild-type cyclin B1. On the other hand, both cyclin B1^{Ala} and S2A-QDA were inactive. These results suggest that Ser-2 phosphorylation is unimportant for cyclin B1 activity, although we cannot rule out the possibility that Ser-2 phosphorylation may play some unidentified role specific to *Xenopus*.

DISCUSSION

Phosphorylation of the CRS Mediates Nuclear Translocation. While previous work from other laboratories has demonstrated nuclear translocation of B-type cyclins as cells approach M phase (16–18), the biological role of such nuclear translocation is undefined and how this is regulated is still unknown. Data presented here provide compelling evidence that phosphorylation at sites within the CRS (13, 15) disrupts the cytoplasmic retention function of this domain, resulting in translocation of cyclin B1 to the nucleus where it acts to promote the $G_2 \rightarrow M$ transition of the cell cycle. Confirming this model, Ser → Glu substitutions at phosphorylation sites within the CRS result in nuclear localization and activation of cyclin B1. Similarly, fusion of a well characterized NLS or a second CRS domain with the Ser phosphorylation sites mutated to Glu can rescue the activity of cyclin B1^{Ala}, which carries Ser → Ala substitutions at the phosphorylation sites

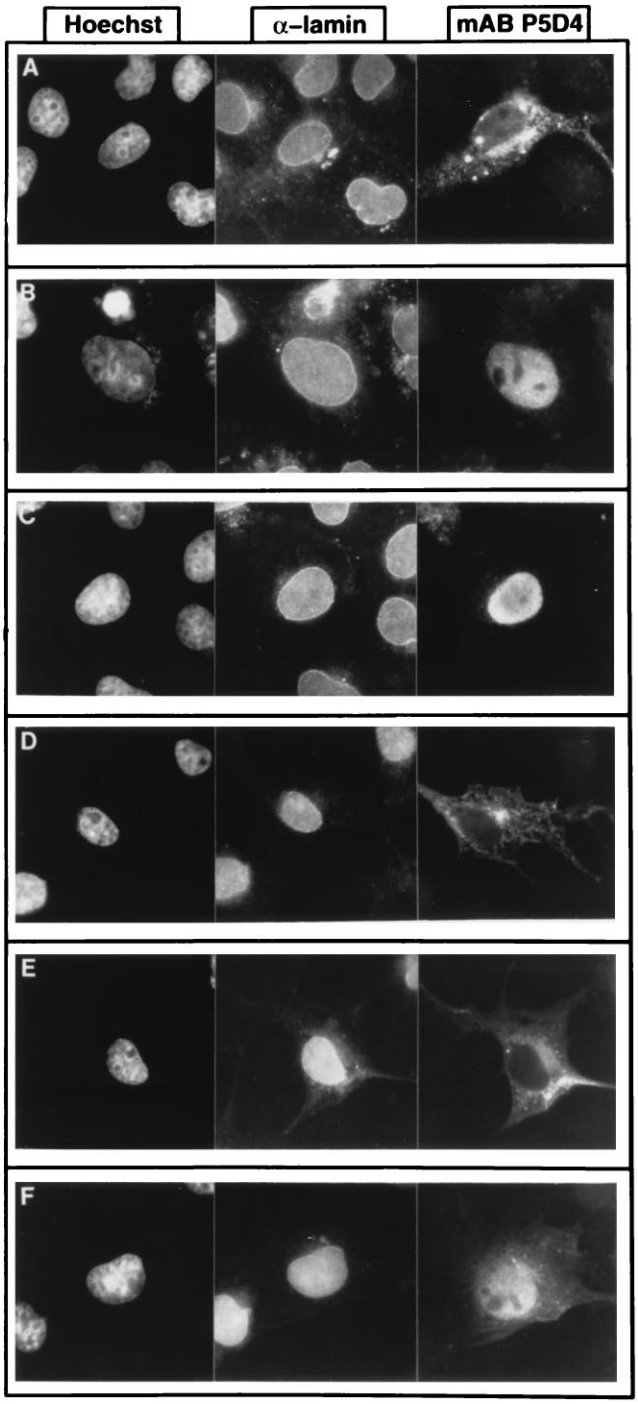

FIG. 6. Localization of cyclin B1 mutants in COS-1 cells by indirect immunofluorescence. Hoechst dye 33342 immunofluorescence (*Left*); α-lamin immunofluorescence (*Center*); and mAb P5D4 immunofluorescence (*Right*) to detect cyclin B1 derivatives: cyclin $B1^{Ala}$ (*A*), cyclin $B1^{Glu}$ (*B*), NLS–$B1^{Ala}$ (*C*), NLS^{mut}–$B1^{Ala}$ (*D*), CRS^{Ala}–$B1^{Ala}$ (*E*), and CRS^{Glu}-$B1^{Glu}$ (*F*).

within the CRS. In contrast, fusion of a second CRS domain with the Ser phosphorylation sites mutated to Ala inactivates cyclin B1. Interestingly, appending CRS^{Ala} to wild-type cyclin B1 (CRS^{Ala}–B1) resulted in complete inactivation (Fig. 5*A*). In contrast, appending CRS^{Ala} to cyclin $B1^{Glu}$ (CRS^{Ala}–$B1^{Glu}$) resulted in only a partial inhibition of activity (Fig. 5*B*). In our previous work (13), we showed that wild-type cyclin B1 is less active than cyclin $B1^{Glu}$ in oocyte maturation assays, probably due to partial phosphorylation of Ser-94, Ser-96, Ser-101, and Ser-113 of exogenously expressed wild-type cyclin B1. Therefore, the nonphosphorylatable CRS^{Ala} may be strong enough to inactivate wild-type cyclin B1, but not strong enough to completely inactivate cyclin $B1^{Glu}$.

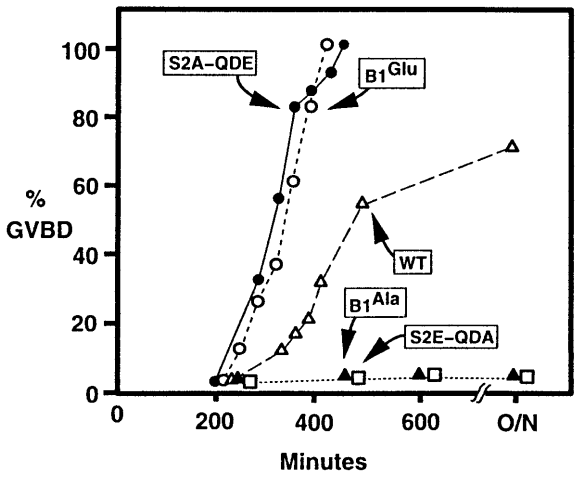

FIG. 7. Kinetics of oocyte maturation in response to cyclin B1 mutants at phosphorylation sites. Procedures for RNA injections and monitoring oocytes were the same as in Fig. 3.

Selective transport of proteins into the nucleus is an important regulatory mechanism (25–28). In many instances, phosphorylation is a critical step in the cytoplasm retention or nuclear translocation, as demonstrated in the cases of mammalian NF-κB (29), lamin B2 (30), yeast SWI5 (31) and simian virus 40 T antigen (32). To our knowledge, this is the first demonstration that nuclear translocation of a key cell cycle component is regulated by phosphorylation. This investigation also provides evidence that the subcellular localization of different cyclins may play a crucial role in regulating cyclin–cyclin-dependent kinase activity (2, 33).

Roles of Cyclin B Nuclear Translocation. Several substrates of MPF, such as nuclear lamins and histone H1, are predominantly nuclear proteins (34–39). Therefore, nuclear translocation of cyclin B may be important in facilitating access of MPF to its nuclear substrates. Another role of nuclear translocation may be to bring pre-MPF together with activators of MPF kinase activity. This is supported by the observation that the MPF activator, cdc25, is detected as a nuclear protein or a cytoplasmine protein which undergoes nuclear translocation during G_2/M transition (40–42).

Where Is the Nuclear Localization Signal? There is no putative NLS in cyclin B1. Although observations made in this investigation might suggest that the phosphorylated CRS domain functions as a NLS itself, we do not believe this to be the case. Fusion of the CRS^{Glu} domain to the protein pyruvate kinase, often used as a reporter to characterize nuclear localization, did not result in nuclear localization of the reporter protein (data not shown). How phosphorylation of cyclin B1 leads to its nuclear translocation is still unclear. One explanation may be that phosphorylation within the CRS may allow this domain, acting in concert with another portion of cyclin B1, to form a functional NLS. Alternatively, phosphorylation within the CRS could allow cyclin B1 to undergo piggyback transport to the nucleus by binding to another protein. We are currently investigating these possibilities.

Does MPF Autophosphorylation Regulate Its Nuclear Translocation? One mechanism leading to cyclin B phosphorylation and nuclear translocation may be the autophosphorylation of MPF itself. Active MPF has been shown to autophosphorylate its cyclin subunit, either as part of MPF or if supplied as an exogenous substrate (43, 44). *In vitro*, purified MPF is able to phosphorylate Ser-94 and Ser-96 in cyclin B1 and Ser-90 in cyclin B2 (14). It is certainly possible that the

autocatalytic activation of MPF not only includes activation of cdc25 and inactivation of wee1 by MPF (45), but also involves promoting its nuclear translocation by autophosphorylation of cyclin B.

Mitogen-Activating Protein (MAP) Kinase and Casein Kinase II: Possible Cyclin Kinases. Previously, we have shown that phosphorylation of cyclin B1 either at Ser-94 and Ser-96 or at Ser-101 and Ser-113 is sufficient for its biological activity, suggesting that these phosphorylation sites are redundant (13). MAP kinase represents one candidate cyclin kinase that has been shown to phosphorylate Ser-94 and/or Ser-96 in cyclin B1 and Ser-90 in cyclin B2 *in vitro* (14). During *Xenopus* oocyte maturation, phosphorylation of cyclin B2 temporally correlates with MAP kinase activation (46). Both Ser-101 and Ser-113 in cyclin B1 are followed by multiple acidic amino acids, which resemble the consensus phosphorylation sites of casein kinase II (47). Thus, there could be two separate pathways regulating B-type cyclin nuclear translocation. One pathway might involve activation of the MAP kinase pathway leading to phosphorylation of cyclin B at Ser-94 and Ser-96. A second, and possibly redundant, pathway could involve casein kinase II or a related protein kinase that phosphorylates Ser-101 and Ser-113, resulting in nuclear translocation of B-type cyclins. In support of this, casein kinase II is active during M phase (48–50).

Because the Ser phosphorylation sites within the CRS are conserved among B-type cyclins in higher eukaryotes (13, 15), it seems likely that the role of phosphorylation in regulating cyclin B localization will emerge as a general feature of cell cycle control. Further elucidation of cyclin B nuclear translocation and identification of physiological cyclin kinases and phosphatases will contribute greatly to our understanding of cell cycle regulation.

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1. Nigg, E. A. (1995) *BioEssays* **17,** 471–480.
2. Doree, M. & Galas, S. (1994) *FASEB J.* **8,** 1114–1121.
3. Ducommun, B., Brambilla, P., Felix, M.-A., Franza, B. R., Karsenti, E. & Draetta, G. (1991) *EMBO J.* **10,** 3311–3319.
4. Gould, K. L., Moreno, S., Owen, D. J., Sazer, S. & Nurse, P. (1991) *EMBO J.* **10,** 3297–3309.
5. Krek, W. & Nigg, E. A. (1991) *EMBO J.* **10,** 305–316.
6. Krek, W. & Nigg, E. A. (1991) *EMBO J.* **10,** 3331–3341.
7. Norbury, C., Blow, J. & Nurse, P. (1991) *EMBO J.* **10,** 3321–3329.
8. Pickham, K. M., Meyer, A. N., Li, J. & Donoghue, D. J. (1992) *Mol. Cell. Biol.* **12,** 3192–3203.
9. Solomon, M. J., Lee, T. & Kirschner, M. W. (1992) *Mol. Biol. Cell* **3,** 13–27.
10. Pines, J. & Hunter, T. (1989) *Cell* **58,** 833–846.
11. Meijer, L., Arion, D., Golsteyn, R., Pines, J., Brizuela, L., Hunt, T. & Beach, D. (1989) *EMBO J.* **8,** 2275–2282.
12. Gautier, J. & Maller, J. L. (1991) *EMBO J.* **10,** 177–182.
13. Li, J., Meyer, A. N. & Donoghue, D. J. (1995) *Mol. Biol. Cell* **6,** 1111–1124.
14. Izumi, T. & Maller, J. L. (1991) *Mol. Cell. Biol.* **11,** 3860–3867.
15. Pines, J. & Hunter, T. (1994) *EMBO J.* **13,** 3772–3781.
16. Gallant, P. & Nigg, E. A. (1992) *J. Cell Biol.* **117,** 213–224.
17. Ookata, K., Hisanaga, S., Okano, T., Tachibana, K. & Kishimoto, T. (1992) *EMBO J.* **11,** 1763–1772.
18. Pines, J. & Hunter, T. (1991) *J. Cell Biol.* **115,** 1–17.
19. Feldherr, C. M. & Richmond, P. A. (1978) *Methods Cell Biol.* **17,** 75–79.
20. Robbins, J., Dilworth, S. M., Laskey, R. A. & Dingwall, D. (1991) *Cell* **64,** 615–623.
21. Glotzer, M., Murray, A. W. & Kirschner, M. W. (1991) *Nature (London)* **349,** 132–138.
22. Kreis, T. E. & Lodish, H. F. (1986) *Cell* **46,** 929–937.
23. Minshull, J., Blow, J. J. & Hunt, T. (1989) *Cell* **56,** 947–956.
24. Kozak, M. (1986) *Cell* **44,** 283–292.
25. Hunt, T. (1989) *Cell* **59,** 949–951.
26. Nigg, E. A., Baeuerle, P. A. & Luhrmann, R. (1991) *Cell* **66,** 15–22.
27. Silver, P. A. (1991) *Cell* **64,** 489–497.
28. Vandromme, M., Gauthierrouviere, C., Lamb, N. & Fernandez, A. (1996) *Trends Biochem. Sci.* **21,** 59–64.
29. Kerr, L. D., Inoue, J., Davis, Link, E., Baeuerle, P. A., Bose, H. R. & Verma, I. M. (1991) *Genes Dev.* **5,** 1464–1476.
30. Hennekes, H., Peter, M., Weber, K. & Nigg, E. A. (1993) *J. Cell Biol.* **120,** 1293–304.
31. Moll, T., Tebb, G., Surana, U., Robitsch, H. & Nasmyth, K. (1991) *Cell* **66,** 743–758.
32. Jans, D. A., Ackermann, M. J., Bischoff, J. R., Beach, D. H. & Peters, R. (1991) *J. Cell Biol.* **115,** 1203–1212.
33. Nigg, E. A. (1993) *Curr. Opin. Cell Biol.* **5,** 187–193.
34. Chambers, T. C. & Langan, T. A. (1990) *J. Biol. Chem.* **265,** 16940–16947.
35. Jerzmanowski, A. & Cole, R. D. (1992) *J. Biol. Chem.* **267,** 8514–8520.
36. Ohsumi, K., Katagiri, C. & Kishimoto, T. (1993) *Science* **262,** 2033–2035.
37. Heald, R. & McKeon, F. (1990) *Cell* **61,** 579–589.
38. Peter, M., Nakagawa, J., Doree, M., Labbe, J. C. & Nigg, E. A. (1990) *Cell* **61,** 591–602.
39. Ward, G. E. & Kirschner, M. W. (1990) *Cell* **61,** 561–577.
40. Heald, R., McLoughlin, M. & McKeon, F. (1993) *Cell* **74,** 463–474.
41. Girard, F., Strausfeld, U., Cavadore, J. C., Russell, P., Fernandez, A. & Lamb, N. J. (1992) *J. Cell Biol.* **118,** 785–794.
42. Millar, J. B., Blevitt, J., Gerace, L., Sadhu, K., Featherstone, C. & Russell, P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10500–10504.
43. Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T. & Maller, J. L. (1990) *Cell* **60,** 487–494.
44. Lohka, M. J., Hayes, M. K. & Maller, J. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3009–3013.
45. Coleman, T. R. & Dunphy, W. G. (1994) *Curr. Opin. Cell Biol.* **6,** 877–882.
46. Kobayashi, H., Golsteyn, R., Poon, R., Stewart, E., Gannon, J., Minshull, J., Smith, R. & Hunt, T. (1991) *Cold Spring Harbor Symp. Quant. Biol.* **56,** 437–447.
47. Pinna, L. A. (1990) *Biochim. Biophys. Acta* **1054,** 267–284.
48. Litchfield, D. W., Luscher, B., Lozeman, F. J., Eisenmann, R. N. & Krebs, E. G. (1992) *J. Biol. Chem.* **267,** 13943–13951.
49. Hanna, D. E., Rethinaswamy, A. & Glover, C. V. (1995) *J. Biol. Chem.* **270,** 25905–25914.
50. Cardenas, M. E., Dang, Q., Glover, C. V. & Gasser, S. M. (1992) *EMBO J.* **11,** 1785–1796.