Entry into mitosis is regulated by the Cdc2 kinase complexed to B-type cyclins. We and others recently reported that cyclin B1/Cdc2 complexes, which appear to be constitutively cytoplasmic during interphase, actually shuttle continually into and out of the nucleus, with the rate of nuclear export exceeding the import rate (1–5). At the time of entry into mitosis, the import rate is increased, whereas the export rate is decreased, leading to rapid nuclear accumulation of Cdc2/cyclin B1. Although it has recently been reported that phosphorylation of 4 serines within cyclin B1 promotes the rapid nuclear translocation of Cdc2/cyclin B1 at G2/M, the role that individual phosphorylation sites play in this process has not been examined (3, 4). We report here that phosphorylation of a single serine residue (Ser113 of this paper) is required for the accelerated nuclear import of cyclin B1. This serine lies directly within the cyclin B1 nuclear export sequence and, when phosphorylated, prevents binding of the nuclear export factor, CRM1. In contrast, analysis of phosphorylation site mutants suggests that coordinate phosphorylation of all 4 serines (94, 96, 101, and 113) is required for the accelerated nuclear import of cyclin B1/Cdc2 characteristic of G2/M. Additionally, binding of cyclin B1 to importin-β, the factor known to be responsible for the slow interphase nuclear entry of cyclin B1, appears to be unaffected by the phosphorylation state of cyclin B. These data suggest that a distinct import factor must be recruited to enhance nuclear entry of Cdc2/cyclin B1 at the G2/M transition.

In all eukaryotes studied to date, the onset of both mitosis and meiosis is regulated by the activation of MPF (maturation-promoting factor), a heterodimeric complex that contains the serine/threonine kinase Cdc2 and a B-type cyclin. Coordination of the G2/M transition relies upon tight regulation of the kinase activity of Cdc2/cyclin B. During interphase, cyclin B is synthesized and forms complexes with the inactive Cdc2 monomer. Phosphorylation of complexed Cdc2 at two sites, Thr14 and Tyr15, by the inhibitory kinases Wee1 and Myt1 keeps Cdc2 inactive until the time of entry into mitosis (5–13). At the G2/M transition, dephosphorylation of Cdc2 by the dual specificity phosphatase, Cdc25, promotes Cdc2/cyclin B activation, thus driving cells into mitosis (14–17).

The biological function of Cdc2/cyclin B is regulated not only at the level of enzymatic activity but also by changes in subcellular localization. During interphase, Cdc2/cyclin B kinase complexes are predominantly cytoplasmic; at the onset of mitosis, they transit precipitously to the nucleus, presumably to catalyze the nuclear events of mitosis (18). Cyclin B2, in contrast, remains cytoplasmic throughout the cell cycle (19). By deletion analysis, the region of cyclin B1 responsible for its interphase cytoplasmic localization maps to a domain near the N terminus of the protein termed the cytoplasmic retention sequence (CRS)1 (19). Recently, we and others reported that the CRS of cyclin B1 contains an autonomous nuclear export sequence that mediates the nuclear export of cyclin B1 through binding of the nuclear transport factor CRM1 (1–3). Because impeding nuclear export through use of the CRM1 inhibitor leptomycin B led to the marked nuclear accumulation of cyclin B1, we concluded that the seemingly static cytoplasmic localization of cyclin B1 during interphase actually reflects a balance of ongoing nuclear import and more rapid nuclear export.

At the onset of mitosis, cyclin B1 is phosphorylated on several sites, among which four conserved serines (Ser94, Ser96, Ser101, and Ser113) within the CRS have been proposed to play a role in mediating the biological activity of cyclin B1 (3, 20). In Xenopus oocytes, mutation of all four serines (to Ala) to abolish phosphorylation diminished the ability of this mutant to induce oocyte maturation (the G2/M transition) (20). In Cos cells, phosphorylation-deficient cyclin B1 protein is cytoplasmic; in contrast, mutation of all four serines to mimic constitutive phosphorylation (Ser to Glu) drives cyclin B1 into the nucleus. Moreover, appending a strong nuclear localization signal (NLS) to the phosphorylation-deficient cyclin B1 restored its ability to promote oocyte maturation (4). Consistent with these observations, we reported that mutation of all four serines to Glu impaired the ability of the CRS to bind to the nuclear export factor CRM1 and prevented cyclin B1 nuclear export, thus facilitating nuclear accumulation of cyclin B1 complexes at the G2/M transition (3).

Although cyclin B1 complexes appear to transit into the nucleus at a continually slow rate during interphase, nuclear import of Cdc2/cyclin B1 rapidly accelerates at the G2/M transition. As reported recently by Pines and colleagues (21),
changing all of the serine residues within the CRS to Ala impedes nuclear import of cyclin B1, whereas changing them all to Glu accelerates import, consistent with a role for phosphorylation in both inhibiting nuclear export and increasing nuclear import of cyclin B1. In this report, we further investigate the role of cyclin B1 phosphorylation in nuclear transport.

Specifically, we were interested in determining whether all four phosphorylation sites within the CRS must act in concert to regulate cyclin B1 trafficking and whether the nuclear import and export processes were controlled by distinct phosphorylation sites. By analyzing CRM1 binding and nuclear transport properties of single and multiple site mutants of cyclin B1 within the CRS, we have found that nuclear export of cyclin B1 is controlled exclusively through phosphorylation of Ser113 (Xenopus numbering), whereas phosphorylation of all four serine residues within the CRS is required to accelerate nuclear import. This offers the opportunity for combinatorial control of Cdc2/cyclin B1 localization by distinct kinases acting at distinct phosphorylation sites, allowing multiple cellular inputs into the regulation of cyclin B1/Cdc2 localization.

**EXPERIMENTAL PROCEDURES**

In Vitro Translation—Xenopus cyclin B1 wild type, Ala, and Glu mutants were kindly provided by Dr. D. Donoghue (University of California, San Diego) in the vector SP64T, containing cyclin variants cloned downstream of an SP6 promoter. 35S-Labeled proteins were produced by use of the SP6-coupled TNT reticulocyte system (Promega) according to manufacturer’s instructions.

Preparation of Xenopus Oocyte and Egg Extracts—Oocyte extracts were prepared as described previously (22). Interphase egg extracts and mitotic extracts were prepared according to the protocols of Smythe and Newport (23).

Oocyte Microinjection and Subfractionation—Stage VI oocytes of Xenopus laevis were prepared for microinjection, as described (24). Oocytes were put in individual wells of the 96-well plate, oriented with animal pole upward. The plates were placed on top of centrifuge bucket adaptors in Beckman table top and spun for 10 min at 1000 × g at 18 °C. This step enabled the nucleus to move closer to the animal pole, and sometimes a white spot (nucleus) can be visualized in the animal pole region. Eight to ten nanoliters of protein sample was injected into oocyte nuclei. Two injection controls for nuclear integrity were used: 14C-labeled bovine serum albumin (Amersham Pharmacia Biotech) and 5 ng/ml of 1-thio-β-galactoside, which all of the serine phosphorylation sites had been changed from the mutation site to the C-terminal end. A full-length mutant clone was generated with an additional round of PCR, using a mixture of the N- and C-terminal encoding DNA fragments as templates for PCR, with the original 5’ and 3’ primers. The full-length product was subcloned into the pSP64T vector. For individual mutants, their mutagenic primers are listed: S94A/3E, 5′-GGCTGAGGTTGACCCGGCG-CCGACGCAAATGGAAC-3′; S96A/3E, 5′-GGTTGAAACCAGGGAAA- CGCCGCGCACTGAAAGAAGG-3′; S101A/3E, 5′-CAGAGCCAATTGGAACGCGGAAACCACTGAAAGAAGG-3′; and S113A/5E, 5′-GC TicGTGCGCTTCATTGACATCGG-3′. For construction of the S113E mutant of cyclin B1, the wild type cyclin B1 cDNA in pSP64T was used as the template, and the PCR-based mutagenesis method was used as described above. The mutagenic primer was 5′-GGCTGCGGAGGGTTTCGAGATTCCATCTCAAGC-3′. All mutations were confirmed by DNA sequencing.

Expression and Purification of Recombinant GST Fusion Proteins—All constructs were expressed in Topp3 E. coli (Stratagene). To increase solubility of recombinant proteins, bacteria were grown to OD of 0.5 at 37 °C and then shifted to 18 °C. 0.4 ml isopropyl-1-thio-β-D-galactopyranoside was added to induce protein expression at 18 °C overnight. Bacteria were pelleted and resuspended in lysis buffer (50 mM Tris, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride). 1% Triton-X and 300 mM NaCl were added to increase protein solubility. Cells were lysed twice using a French Press and spun at 17,000 × g for 30 min. The supernatants were diluted 1:1 with buffer (10 mM Heps, pH 8.0, and 1 mM DTT) to reduce the Triton concentration and incubated with glutathione-Sepharose beads at 4 °C for 1–2 h. Beads were pelleted, washed with buffer (10 mM Heps, pH 8.0, 300 mM NaCl, and 1 mM DTT), and kept in storage buffer (10 mM Heps, pH 8.0, 50% glycerol, and 1 mM DTT) at −20 °C.

Pull-down Experiments—GST fusion proteins were coupled to glutathione-Sepharose beads and blocked with boiled fetal bovine serum or 1% casamino acids plus 0.1% Tween 20 to block nonspecific interactions of proteins with the Sepharose beads.

RESULTS

Nuclear Export of Cyclin B1 Is Controlled by Phosphorylation of Ser113—As described above, we have shown previously that phosphorylation of four conserved serines within the CRS region of cyclin B1 can regulate its nuclear export (3). Specifically, mutation of all four serines to Glu mimics constitutive phosphorylation because all four of these serines (Ser34, Ser256, Ser311, and Ser315) had been mapped as phosphorylation sites in vitro (4). Hence, in these experiments, for simplicity, we assume that each of these sites is a potential target for regulation of nuclear trafficking, where the simplest scenario, if phosphorylation of a single site is required to prevent the cyclin B1-CRM1 interaction, is that site nonphosphorylatable (through mutation to Ala) should permit interaction with CRM1, even if all of the other CRS serine residues are phosphorylated (or mutated to Glu to mimic constitutive phosphorylation). Therefore, starting with a GST-CRS construct in which all of the serine phosphorylation sites had been changed to Glu, we mutated each site individually back to Ala and examined the resulting panel of mutant CRS regions for their

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| SERINE PHOSPHORYLATION MUTANTS | XENOPUS CYCLIN B1 4-Glu MUTANT DNA IN pSP64T VECT | GENERATED A SECOND PCR PRODUCT USING THE C-TERMINAL PRIMER AND THE CORRESPONDING PHOSPHORYLED PROTEIN, PRODUCING A DNA FRAGMENT EXTENDING FROM THE MUTATION SITE TO THE C-TERMINAL END. A FULL-LENGTH MUTANT CHAIN CAN BE GENERATED WITH AN ADDITIONAL ROUND OF PCR, USING A MIXTURE OF THE N- AND C-TERMINAL ENCODING DNA FRAGMENTS AS TEMPLATES FOR PCR, WITH THE ORIGINAL 5’ AND 3’ PRIMERS. THE FULL-LENGTH PRODUCT WAS SUBCLONED INTO THE pSP64T VECTOR. FOR INDIVIDUAL MUTANTS, THEIR MUTAGENIC PRIMERS ARE LISTED: S94A/3E, 5′-GGCTGAGGTTGACCCGGCG-CCGACGCAAATGGAAC-3′; S96A/3E, 5′-GGTTGAAACCAGGGAAA- CGCCGCGCACTGAAAGAAGG-3′; S101A/3E, 5′-CAGAGCCAATTGGAACGCGGAAACCACTGAAAGAAGG-3′; AND S113A/5E, 5′-GC TicGTGCGCTTCATTGACATCGG-3′. FOR CONSTRUCTION OF THE S113E MUTANT OF CYCLIN B1, THE WILD TYPE CYCLIN B1 cDNA IN pSP64T WAS USED AS THE TEMPLATE, AND THE PCR-BASED MUTAGENESIS METHOD WAS USED AS DESCRIBED ABOVE. THE MUTAGENIC PRIMER WAS 5′-GGCTGCGGAGGGTTTCGAGATTCCATCTCAAGC-3′. ALL MUTATIONS WERE CONFIRMED BY DNA SEQUENCING.

Expression and Purification of Recombinant GST Fusion Proteins—All constructs were expressed in Topp3 E. coli (Stratagene). To increase solubility of recombinant proteins, bacteria were grown to OD of 0.5 at 37 °C and then shifted to 18 °C. 0.4 ml isopropyl-1-thio-β-D-galactopyranoside was added to induce protein expression at 18 °C overnight. Bacteria were pelleted and resuspended in lysis buffer (50 mM Tris, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride). 1% Triton-X and 300 mM NaCl were added to increase protein solubility. Cells were lysed twice using a French Press and spun at 17,000 × g for 30 min. The supernatants were diluted 1:1 with buffer (10 mM Heps, pH 8.0, and 1 mM DTT) to reduce the Triton concentration and incubated with glutathione-Sepharose beads at 4 °C for 1–2 h. Beads were pelleted, washed with buffer (10 mM Heps, pH 8.0, 300 mM NaCl, and 1 mM DTT), and kept in storage buffer (10 mM Heps, pH 8.0, 50% glycerol, and 1 mM DTT) at −20 °C.

Pull-down Experiments—GST fusion proteins were coupled to glutathione-Sepharose beads and blocked with boiled fetal bovine serum or 1% casamino acids plus 0.1% Tween 20 to block nonspecific interactions of proteins with the Sepharose beads. The beads were then incubated with either oocyte extract or in vitro translated protein in boiled fetal bovine serum (or 1% casamino acids plus 0.1% Tween 20) at 4 °C for an hour. The beads were then washed with EB buffer, and the binding proteins were resolved by SDS-PAGE followed by immunoblotting or autoradiography.

RESULTS

Nuclear Export of Cyclin B1 Is Controlled by Phosphorylation of Ser113—As described above, we have shown previously that phosphorylation of four conserved serines within the CRS region of cyclin B1 can regulate its nuclear export (3). Specifically, mutation of all four serines to Glu mimics constitutive phosphorylation because all four of these serines (Ser34, Ser256, Ser311, and Ser315) had been mapped as phosphorylation sites in vitro (4). Hence, in these experiments, for simplicity, we assume that each of these sites is a potential target for regulation of nuclear trafficking, where the simplest scenario, if phosphorylation of a single site is required to prevent the cyclin B1-CRM1 interaction, is that site nonphosphorylatable (through mutation to Ala) should permit interaction with CRM1, even if all of the other CRS serine residues are phosphorylated (or mutated to Glu to mimic constitutive phosphorylation). Therefore, starting with a GST-CRS construct in which all of the serine phosphorylation sites had been changed to Glu, we mutated each site individually back to Ala and examined the resulting panel of mutant CRS regions for their...
ability to bind CRM1. As shown in Fig. 1, changing Ser113, but not any other site, to Ala (S113A/3E) conferred on cyclin B1 the ability to bind to CRM1. These data suggest that phosphorylation of Ser113 at the G2/M transition blocks binding of cyclin B1, and that it very efficiently prevented CRM1 binding. These data indicate that phosphorylation of Ser113 is primarily responsible for controlling the cyclin B1-CRM1 interaction.

To determine whether phosphorylation of Ser113 directly modulates cyclin B1 nuclear export, we introduced the S113E and S113A/3E mutations into the full-length wild-type cyclin B1 to compare their nuclear export rates with 4-Glu and wild-type cyclin B1. For this purpose, the mutant and wild-type cyclins were translated in vitro in the presence of [35S]methionine and injected into the nuclei of stage VI oocytes. [35S]-labeled GRP94, which contains neither an NES nor an NLS, was co-injected as a control for maintenance of nuclear integrity during the microinjection and microdissection procedures. At 0, 3, and 6 h after injection, the oocytes were dissected manually into nuclear and cytoplasmic fractions. After identification of successfully injected oocytes (by the presence of the pigmented hemoglobin from the reticulocyte lysate), nuclear and cytoplasmic fractions were analyzed by SDS-PAGE and autoradiography. The presence of labeled protein. In agreement with the CRM1 binding result, we found that introduction of the S113E mutation into full-length cyclin B1 had an effect similar to that of the 4-Glu mutation, significantly reducing the cyclin B1 nuclear export rate (Fig. 2A). Moreover, changing only Ser113 to Ala, in a context where all of the other Ser were changed to Glu (S113A/3E) completely restored cyclin B1 nuclear export (Fig. 2B). It is interesting to note that the S113A/3E mutant exports from nuclei even faster than the wild-type cyclin B1, whose export is slightly retarded by a low basal level of nuclear Ser113-directed kinase activity (Fig. 2B and data not shown). Collectively, these data strongly suggest that nuclear export of cyclin B1 is controlled by phosphorylation of Ser113.

Phosphorylation of All Four Serines within the CRS Is Required for Acceleration of Cyclin B1 Nuclear Import—As noted by Fines and colleagues (1), the slow nuclear import rate observed when cyclin B1 nuclear import is inhibited during interphase can fully account for the rapid nuclear accumulation of cyclin B1 observed at the G2/M transition. Indeed, as they recently reported, phosphorylation within the CRS of human cyclin B1 seems to accelerate its nuclear import, as well as retarding its export (21). To confirm that this was the case in the Xenopus system, we compared the nuclear import rates of the wild-type 4-Glu and 4-Ala mutants by injecting [35S]-labeled cyclin B1 variants into the cytoplasm of oocytes pretreated with leptomycin B to block CRM1-mediated nuclear export. At various time points after injection, the oocytes were dissected into cytoplasmic and nuclear fractions, and the amount of radiolabeled cyclin B1 in each fraction was quantitated. 14C-Labeled bovine serum albumin, which lacks both NES and NLS sequences, was co-injected as a control for maintenance of nuclear integrity during the injection and microdissection procedures. As shown in Fig. 3, changing Ser to Glu to mimic constitutive phosphorylation dramatically accelerated the cyclin B1 nuclear import rate, confirming that phosphorylation within the CRS both inhibits nuclear export and accelerates nuclear import.

To extend these studies and to understand how phosphorylation of individual serines modulates cyclin B1 nuclear import, we again started with the most uncomplicated scenario; if phosphorylation of a single serine is absolutely required to accelerate the nuclear import of cyclin B1 at the G2/M transition, then rendering that site nonphosphorylatable (mutated to Ala) should slow down its import rate to the level of wild type.
cyclin B1, even when all of the other sites are mutated to Glu to mimic constitutive phosphorylation. Therefore, we compared the nuclear import rates of various cyclin B1 mutants, in which each serine was individually mutated to Ala, whereas all of the other serines remained Glu. Surprisingly, we found that rendering any of the four CRS serine residues unphosphorylatable decreased cyclin B1 nuclear import rates to wild type interphase levels (Fig. 4). Therefore, we conclude that phosphorylation of all four serines within the CRS is required to increase the nuclear import rate of cyclin B1 prior to entry into mitosis.

Phosphorylation within the CRS Does Not Regulate Direct Binding to Importin-β—We recently reported that the interphase nuclear import of cyclin B1 is mediated by direct binding to the nuclear transport factor, importin-β (26). It has also been suggested that cyclin B1 can “piggyback” into the nucleus at G2/M by binding to another cyclin protein, cyclin F (25). However, because cyclin F binding to cyclin B1 is reportedly unaffected by phosphorylation of cyclin B1, it seems unlikely that the phosphorylation-induced increase in cyclin B1 nuclear entry is due to enhanced cyclin F interactions (25). Therefore, we sought to determine whether the enhanced import of the 4-Glu mutant and the diminished import of the Ala mutants might reflect changes in the efficiency of the importin-β-cyclin B1 interaction. Accordingly, we incubated radiolabeled, in vitro translated cyclin B1 variants with GST-importin-β immobilized on glutathione-Sepharose and examined the degree to which the CRS mutations affected importin-β binding. In multiple experiments, the characteristic, differential import rates of the various mutants were not reflected in differences in importin binding (Fig. 5). Therefore, phosphorylation does not appear to enhance nuclear import of cyclin B1 by increasing the affinity of cyclin B1 for importin-β.

The Ability of Cyclin B1 to Induce the G2/M Transition Requires Phosphorylation of All Four Serines within the CRS—As reported by Li et al. (4), cyclin B1 bearing a 4-Glu mutant CRS is a much more potent inducer of oocyte maturation (G2/M transition as scored by GVBD) than are the wild type or 4-Ala proteins. To analyze the effects of the individual phosphorylation site mutations, the mutants in which single serines were altered to Ala, whereas all of the other serines were altered to Glu, were tested for their ability to induce GVBD following injection into stage VI G2-arrested oocytes. Consistent with our observation that mutation of any single site to Ala compromised nuclear import, none of the individual mutants were capable of driving the G2/M transition as efficiently as the 4-Glu mutant (Table I). These data strongly suggest that phosphorylation of all four serines within the CRS is not only required for optimal nuclear accumulation of cyclin B1 but is also required for maximally efficient promotion of the G2/M transition.
nucleus. We show in this report that phosphorylation of all four sites within the CRS is required for the rapid nuclear entry of Cdc2/cyclin B1 characteristic of the G2/M transition. In contrast, phosphorylation of a single site (Ser113) suffices to inhibit cyclin B1 nuclear export.

**Phosphorylation Site Effects on Interaction with Nuclear Transport Factors**—The ability of a single site of serine phosphorylation to inhibit cyclin B1 nuclear export is easily explainable given the fact that Ser113 lies within the CRM1-binding nuclear export sequence, immediately adjacent to a hydrophobic residue required for NES activity (2, 3, 21). Phosphorylation of this site introduces a charge that most likely interferes with recognition of the sequence by CRM1. It is not as clear, however, how phosphorylation within the CRS accelerates nuclear import of Cdc2/cyclin B1. Although direct interaction of cyclin B1 with importin-β appears to account for the interphase nuclear import of cyclin B1, it is not clear whether importin-β is involved in the more rapid nuclear entry of cyclin B1 at G2/M (26). That this interaction may indeed be important for G2/M nuclear translocation of cyclin is suggested by the fact that immunodepletion of importin-β from mitotic cytosolic extracts compromises the ability of these extracts to promote cyclin B1 nuclear translocation in a semi-permeabilized cell nuclear transport assay (27). In our assays, there was no discernible difference in the ability of the various phosphorylation site mutants to interact with importin-β. The assays shown in Fig. 5 were, however, performed in buffer with isolated, recombinant importin-β in the absence of ancillary factors. Hence, if importin-β recruits a novel factor responsible for modulating interaction with cyclin B1, it would not be picked up in this particular assay. That being said, we have observed no consistent changes in importin-β binding of these mutants, even when the incubations were performed in the presence of either interphase or mitotic cytosol (data not shown). Similarly, although it has been suggested that cyclin B1 can piggyback into the nucleus through interaction with nuclear-bound cyclin F, this interaction is not modulated by phosphorylation within the CRS (25). Therefore, we suspect either that phosphorylation of cyclin B1 induces interaction with a distinct transport factor other than importin-β or that the altered efficiency of nuclear entry at the G2/M is not controlled at the level of cyclin B1-import factor interactions. Before it was discovered that the CRS region of cyclin B1 provides a binding site for CRM1, it was proposed that the CRS provided an anchoring site for cyclin B1 in the cytoplasm and, in this way, prevented its untimely nuclear entry. One intriguing possibility is that cyclin B1 does, indeed, have a cytoplasmic retention mechanism (in addition to a nuclear export mechanism) and that this mechanism is regulated by phosphorylation within the CRS. Under these circumstances, unphosphorylated cyclin B1 would still have the capacity to interact with transport factors but the “anchor” would in some way physically prevent the rapid nuclear translocation characteristic of mitotic entry. Because leptomycin B treatment of cells does eventually allow the quantitative accumulation of cyclin B1 in the nucleus, such a hypothetical retention mechanism would have to be “leaky” enough to allow slow interphase import or be selective enough to interfere with the import mechanism used at mitosis, without inhibiting the importin-β-mediated interphase import. An alternative, although perhaps unlikely, explanation for the nuclear accumulation of cyclin B1 in the presence of leptomycin B is that a factor required for restricting the nuclear import of unphosphorylated cyclin B1 must itself be continually exported from the nucleus. Such a possibility has also been suggested for the Cdc2 activator Cdc25, whose interphase nuclear import is restricted by binding to 14–3-3 proteins yet rapidly accumulates in nuclei upon leptomycin B treatment (28, 29).

**Phosphorylation of Cyclin B and Entry into Mitosis**—The presence of four phosphorylation sites that must be coordinately controlled to promote nuclear translocation of cyclin B1 offers the possibility for multiple regulatory inputs into cyclin localization. We and others have data to suggest that Ser264 is a bona fide Cdc2 phosphorylation site6 and Ser113 is also a potential Cdc2 site based on sequence consensus (30). Although the kinases responsible for phosphorylation of serines 101 and 113 has not yet been identified, these sites do not fit the consensus for Cdc2-catalyzed phosphorylation. Moreover, the sequences flanking Ser101 and Ser113 are not similar to each other, suggesting that more than one kinase may phosphorylate these sites. Thus, it is seems likely that two or more kinases cooperate to trigger nuclear translocation of the Cdc2/cyclin B1 complex at G2/M. Because all four sites must be phosphorylated for rapid cyclin B1 nuclear import, phosphorylation of cyclin B1 is likely to occur first in the cytoplasm. This is consistent with our previously published observation that the vast majority of

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**Fig. 5. Direct binding of cyclin B1 to importin-β is not affected by the cyclin B1 phosphorylation state.** A, the cyclin B1 mutants analyzed in Fig. 4 were translated in vitro in the presence of [35S]methionine and incubated in phosphate-buffered saline containing 0.1% Tween 20 and 1% casamino acids (as a blocking agent) for 1 h at 4 °C along with GST-importin-β protein linked to glutathione-Sepharose. These beads were pelleted, washed extensively, resolved by SDS-PAGE, and processed for autoradiography. B, quantitation of the data in A. Shown are percentages of input cyclin B1 protein retrieved on the importin-β beads.

**TABLE I**

| Cyclin B1 mutants | Percentage of GVBD |
|-------------------|-------------------|
| Wild type        | 26                |
| 4-Glu            | 100               |
| 4-Ala            | 0                 |
| S113E/3A         | 0                 |
| S94A/3E          | 4                 |
| S96A/3E          | 15                |
| S101A/3E         | 4                 |
| S113A/3E         | 0                 |

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2 H. Song and S. Kornbluth, unpublished observations.
CRS-directed kinase activity is cytoplasmic rather than nuclear. Preliminary data from our laboratory suggest that phosphorylation of Ser^{133} may rely upon prior phosphorylation of one or all of the other serines, providing an additional layer of assurance that nuclear export of Cdc2/cyclin B1 will not be prematurely turned off by Ser^{133} phosphorylation of cyclin B1 shuttling through the nucleus during interphase.

This stringent regulation of cyclin B1 trafficking prevents premature mitotic entry and may be a component of the mechanism that cells use to prevent the onset of mitosis in the presence of damaged or incompletely replicated DNA. It has been reported that Cdc2/cyclin B1 is cytoplasmic in G2-arrested cells bearing damaged DNA (2). Indeed, forcibly localizing Cdc2/cyclin B1 to the nucleus by appending a classical NLS can trigger premature mitotic events in DNA damage-arrested cells harboring a Cdc2 allele insensitive to inhibitory phosphorylation (31). Similarly, Toyoshima et al. (2) reported that a nuclear export-defective cyclin B1 mutant was more effective than wild type cyclin B1 in cooperating with caffeine to override a DNA damage-induced G2 arrest. Given these effects of cyclin B1 subcellular localization on checkpoint function, it will be interesting, in the future, to determine whether G2/M checkpoint pathways directly modulate the phosphorylation state of the cyclin B1 CRS.

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