Maintenance of G2 arrest in the Xenopus oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import

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Cdc2–cyclin B1 in the G2-arrested Xenopus oocyte is held inactive by phosphorylation of Cdc2 at two negative regulatory sites, Thr14 and Tyr15. Upon treatment with progesterone, these sites are dephosphorylated by the dual specificity phosphatase, Cdc25, leading to Cdc2–cyclin B1 activation. Whereas maintenance of the G2 arrest depends upon preventing Cdc25-induced Cdc2 dephosphorylation, the mechanisms responsible for keeping Cdc25 in check in these cells have not yet been described. Here we report that Cdc25 in the G2-arrested oocyte is bound to 14-3-3 proteins and that progesterone treatment abrogates this binding. We demonstrate that Cdc25, apparently statically localized in the cytoplasm, is actually capable of shuttling in and out of the oocyte nucleus. Binding of 14-3-3 protein markedly reduces the nuclear import rate of Cdc25, allowing nuclear export mediated by a nuclear export sequence present in the N-terminus of Cdc25 to predominate. If 14-3-3 binding to Cdc25 is prevented while nuclear export is inhibited, the coordinate nuclear accumulation of Cdc25 and Cdc2–cyclin B1 facilitates their mutual activation, thereby promoting oocyte maturation.

Keywords: 14-3-3 protein/Cdc2–cyclin B1/Cdc25/oocyte maturation/Xenopus

Introduction

Xenopus oocytes are physiologically arrested in G2 of meiosis I. Upon treatment with progesterone, these oocytes undergo meiotic maturation, leading to breakdown of the nuclear envelope (germinal vesicle breakdown; GVBD), chromosome condensation and spindle formation. Whereas many of the molecular details of this process have yet to be elucidated, it is clear that obligatory steps in progesterone-induced oocyte maturation include translation of mRNA encoding the mos protein kinase, consequent activation of a mitogen-activated protein kinase (MAPK) cascade and activation of maturation promoting factor (MPF), consisting of a Cdc2 kinase catalytic subunit complexed to a B-type cyclin (Masui and Markert, 1971; Sagata et al., 1989; Kaniki and Donoghue, 1991; Nebreda and Hunt, 1993; Shibuya and Ruderman, 1993; Kosako et al., 1994). In the oocyte, Cdc2–cyclin B complexes are stockpiled in an inactive form, poised for progesterone-induced activation. Phosphorylation of the Cdc2 subunit at two negative regulatory sites, Thr14 and Tyr15, is primarily responsible for the inactivity of the stored complexes (Cyrer and Kirschner, 1988; Gautier and Maller, 1991; Kobayashi et al., 1991). In somatic cells, phosphorylation of Tyr15 is catalyzed by two related kinases: a nuclear kinase called Wee1, and a cytoplasmic, membrane-associated kinase called Mnt1 [Mnt1 also phosphorylates Thr14; reviewed in Coleman and Dunphy (1994) and Lew and Kornbluth (1996)]. However, Wee1 appears to be entirely absent from Xenopus oocytes, so Mnt1 is thought to be primarily responsible for phosphorylating Cdc2 in these cells (Murakami and Vande Woude, 1998). It has recently been reported that progesterone treatment leads to inactivation of Mnt1, through a MAPK-induced activation of the kinase p90rsk (Palmer et al., 1998). Mnt1 physically associates with the active hyperphosphorylated form of rsk and phosphorylation of Mnt1 by rsk inhibits Mnt1 activity. Presumably, inactivation of Mnt1 allows the dephosphorylation of Cdc2 Thr14 and Tyr15, leading to MPF activation (Atherton-Fessler et al., 1994; Kornbluth et al., 1994; Mueller et al., 1995). Dephosphorylation of Thr14 and Tyr15 on Cdc2 is catalyzed by the dual (Thr/Tyr) specificity phosphatase, Cdc25 (Duphny and Kumagai, 1991; Gautier et al., 1991; Strausfeld et al., 1991; Millar and Russell, 1992). In somatic cells, Cdc25 alternates between an interphase form with low activity and a hyperphosphorylated mitotic form with high activity. Oocyte maturation is also accompanied by Cdc25 hyperphosphorylation (as evidenced by a shift in the electrophoretic mobility of Cdc25) and activation (Izumi et al., 1992; Kumagai and Duphny, 1992; Hoffmann et al., 1993).

Although physiologically distinct from the G2 arrest of the oocyte, the checkpoint-induced G2 arrest of somatic cells in response to DNA damage or stalled DNA replication also involves the suppression of pre-formed Cdc2–cyclin B complexes through phosphorylation of Cdc2 at Thr14 and Tyr15 (Enoch and Nurse, 1990; Enoch et al., 1992; Smythe and Newport, 1992). Several groups have recently demonstrated that checkpoint-activated kinases phosphorylate Cdc25 at a critical regulatory site (Ser216 of human Cdc25C or Ser287 of Xenopus Cdc25; Peng et al., 1997; Kumagai et al., 1998a; Zeng et al., 1998). This phosphorylation creates a binding site for members of a family of small acidic proteins collectively called 14-3-3 proteins (Peng et al., 1997; Kumagai et al., 1998b; Zeng et al., 1998). Binding by 14-3-3 seems to functionally ‘inactivate’ Cdc25, and is critical for maintaining the checkpoint-induced G2 arrest. Interestingly, 14-3-3 binding does not alter Cdc25 activity assayed in vitro, suggesting that 14-3-3 somehow sequesters Cdc25, perhaps altering its subcellular localization to prevent access of Cdc25 to the Cdc2–cyclin B substrates (Peng et al., 1997).
Factors which modulate the subcellular localization of Cdc2–cyclin B complexes may also contribute to the maintenance of DNA-responsive checkpoint-induced cell-cycle arrest (Jin et al., 1998; Toyoshima et al., 1998). We and others have recently shown that Cdc2–cyclin B1 complexes, which appear to be statically localized to the cytoplasm during interphase, actually shuttle continuously in and out of the nucleus, where they might in some way ‘read’ the status of the DNA (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). During interphase, nuclear export of cyclin B1, mediated by the nuclear export receptor CRM1, predominates over nuclear import. However, at the G2/M transition, phosphorylation of cyclin B1 in the region of its nuclear export sequence (NES) prevents nuclear export, thereby fostering the nuclear accumulation of Cdc2–cyclin B1 required for the nuclear events of mitosis (Li et al., 1995, 1997; Yang et al., 1998). Although it has not been demonstrated that the DNA damage or replication checkpoints directly regulate cyclin B localization, there are data to suggest that this may be the case. Indeed, forcibly localizing cyclin B1 to the nucleus by appending a strong nuclear localization sequence, or by inactivating its nuclear export, compromises checkpoint function (Jin et al., 1998; Toyoshima et al., 1998).

In this report, we show that Cdc25 in the G2-arrested Xenopus oocyte is complexed to 14-3-3 proteins, and that this binding is abrogated by progesterone treatment. Further, we demonstrate that the apparently cytoplasmic Xenopus Cdc25 contains an intrinsic CRM1-binding nuclear export sequence and can, like cyclin B1, shuttle in and out of the nucleus. Mutation of Cdc25 to prevent 14-3-3 binding resulted in a dramatic increase in the nuclear import rate of Cdc25, without markedly perturbing its nuclear export rate. These findings indicate that the G2 arrest of the oocyte employs similar strategies to those operating in response to checkpoint controls, and provides a mechanistic basis for the functional inhibition of Cdc25 by 14-3-3 proteins.

Results

To determine whether Xenopus Cdc25 bound 14-3-3 proteins in the G2-arrested oocyte, we prepared extracts from either untreated oocytes or oocytes after progesterone treatment. When anti-Cdc25 immunoprecipitates from these extracts were immunoblotted with antisera directed against 14-3-3 ε, the predominant Cdc25-binding variant in interphase Xenopus egg extracts (Kumagai et al., 1998), we found that the G2-arrested oocytes contained Cdc25–14-3-3 complexes, which were no longer detectable at the time of progesterone-induced GVBD (Figure 1). These data suggested the possibility that Cdc25 binding by 14-3-3 proteins might contribute to the maintenance of G2 arrest in the oocyte.

Cytoplasmic accumulation of Cdc25 reflects continuous nuclear import and rapid re-export

Since binding of Cdc25 by 14-3-3 proteins does not appear to alter Cdc25 enzymatic activity, we wished to explore the possibility that 14-3-3 binding might regulate the subcellular localization of Cdc25 in the oocyte. Like cyclin B1, Cdc25C is cytoplasmic during interphase and enters the nucleus just prior to mitosis, at least in some somatic cells (Seki et al., 1992). Also like cyclin B1, Cdc25 is almost entirely cytoplasmic in the G2-arrested Xenopus oocyte (Izumi et al., 1992; Yang et al., 1998). We have recently shown that cyclin B1 in fact shuttles in and out of the germinal vesicle (GV), leading us to suspect that the apparently static localization of Cdc25 belied its ability to shuttle in and out of nuclei. To test this possibility, we monitored Cdc25 localization after treating oocytes with leptomycin B, an inhibitor of CRM1-mediated nuclear export (Fornerod et al., 1997; Neville et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997; Wolff et al., 1997). Anti-Cdc25 immunoblotting of nuclear and cytoplasmic fractions from manually dissected oocytes revealed that inhibiting nuclear export promoted a striking nuclear accumulation of Cdc25, which occurred even faster than cyclin B1 nuclear accumulation in the same system (Figure 2A and B; Yang et al., 1998). Thus, as for cyclin B1, cytoplasmic localization of Cdc25 results from ongoing nuclear import and more rapid re-export.

Xenopus Cdc25 is a nuclear export substrate in both the presence and absence of 14-3-3 binding

To determine if 14-3-3 binding affected Cdc25 localization, we wished to examine the consequences of this binding for Cdc25 nuclear import and export rates. To confirm that Cdc25 could, indeed, serve as a nuclear export substrate, we injected 35S-labeled in vitro-translated Cdc25 into oocyte nuclei, and then manually dissected the oocytes into cytoplasmic and nuclear fractions at various times after injection. Within 60 min, virtually all of the Cdc25 had been exported from nuclei (Figure 2C). When we injected nuclei from the same batch of oocytes with Cdc25 protein which had been mutated to abrogate 14-3-3 binding (Cdc25 S287A), we found that the mutant Cdc25 appeared to exit nuclei at a rate only marginally slower than the wild-type (a point we will return to below).

Identification of a CRM1-binding NES in Cdc25

The inhibition of Cdc25 nuclear export by leptomycin-B suggested that Cdc25 export was mediated by CRM1. We produced recombinant derivatives of Cdc25 in Escherichia coli to examine whether they could bind to CRM1 in oocyte extracts. First, we fused only the N-terminal 322 amino acids or the C-terminal 228 amino acids of Cdc25 to glutathione S-transferase (GST), immobilized...
the fusion proteins on glutathione–Sepharose and incubated these in oocyte extract as a source of CRM1. After extensive washing, proteins remaining bound to the beads were resolved by SDS–PAGE and immunoblotted with CRM1 antisera. As shown in Figure 3A, a protein containing the N-terminal 322 amino acids of Cdc25 was able to bind CRM1, whereas a protein containing the C-terminal 228 amino acids of Cdc25 was not. Furthermore, a Cdc25 N-terminal fragment containing the S287A mutation bound equally well to CRM1 despite its inability to bind 14-3-3 (Figure 3B). This suggests that the Cdc25–CRM1 interaction is unaffected by 14-3-3, consistent with the observed similar export rates of the full-length wild-type and S287A mutant Cdc25 proteins.

To localize the Cdc25 NES more precisely, we fused GST to successively smaller portions of the N-terminal fragment, serially deleting regions of the protein, starting from its C-terminus (aa 322; Figure 3C). As above, these fusion proteins were tested for their ability to retrieve CRM1 from oocyte extracts. We found that all of the fusion proteins examined, including one containing only the first 100 amino acids of Cdc25, were able to interact with CRM1 (Figure 3D). A scan of the first 100 amino acids revealed a single sequence, 47LTPVTDLAV55, matching the consensus sequence for a CRM1-binding, leucine-rich NES (Bogerd et al., 1996). To determine whether this sequence affected nuclear export of Cdc25, we mutated the last two required hydrophobic residues, L53 and V55, to Ala (Figure 4A). This mutant protein was not detectably exported following microinjection into oocyte nuclei (Figure 4B and C) and a recombinant truncated Cdc25 protein bearing the same mutations bound well to 14-3-3 protein, but could not bind to CRM1 (Figure 4D). These data strongly suggest that the mutated residues lie within a functional NES which is responsible for Xenopus Cdc25 nuclear export.

**Cdc25 S287A induces GVBD in leptomycin-B-treated oocytes**

Since 14-3-3 binding did not appear to modulate the nuclear export rate of Cdc25, we wished to determine whether the nuclear import rate of Cdc25 might be affected. To this end, we injected radiolabeled wild-type or S287A Cdc25 proteins into the cytoplasm of oocytes which had been pre-treated with leptomycin B to prevent re-export. Intriguingly, under these conditions the S287A protein induced MPF activation (assayed as histone H1-directed kinase activity) and GVBD ~6 h after injection (Figure 5A). This effect required leptomycin B treatment and did not occur with the wild-type Cdc25. Since we injected trace quantities of radiolabeled protein (<2% of endogenous Cdc25), this result indicates that the S287A Cdc25 mutant has considerably increased biological potency compared with the wild-type, in agreement with the recently described relative potency of similar Cdc25 mutants in overcoming a checkpoint arrest in somatic
Fig. 3. The N-terminal region of *Xenopus* Cdc25 contains a CRM1-binding NES, which can bind to CRM1 in the presence and absence of 14-3-3 binding. (A) N-terminal (aa. 1–322) or C-terminal (aa. 323–550) fragments of Cdc25 were fused to GST and coupled to glutathione–Sepharose beads. Twenty microliters of these resins or control GST resin were incubated in 100 μl of interphase extract for 1 h. The beads were then pelleted and washed five times with egg lysis buffer. The bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-human CRM1 antibody. (B) The N-terminal (aa 1–322) fragments of wild-type or S287A mutant Cdc25 proteins were fused to GST and coupled to glutathione–Sepharose beads. The pull-down assay was performed as in (A), and the bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-CRM1 and anti-14-3-3 ε antibody. (C) The truncated proteins derived from the N-terminal fragment of Cdc25 were boiled in SDS sample buffer and eluted proteins were subjected to SDS–PAGE and Coomassie-blue staining, to show the molecular weights of the deleted fusion proteins. (D) Twenty microliters of the resins shown in (C) were used for pull-down assays as described in (A). The bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-human CRM1 antibody.

Fig. 4. Identification of the CRM1-binding NES in *Xenopus* Cdc25. (A) Residues 47–55 of *Xenopus* Cdc25 comprise a putative NES. Asterisks indicate consensus leucine-rich residues. L53 and V55 were mutated to Ala to create the Cdc25:NES-null protein. (B) 35S-labeled Cdc25:WT or Cdc25:NES-null proteins were coinjected into oocyte nuclei with GRP94 control protein. Oocytes were dissected 0 or 1 h later and proteins were extracted and analyzed by SDS–PAGE and autoradiography. Percent remaining in the nucleus by 2 h (not shown), the NES-null protein had still not exited from nuclei. (C) The bar graph represents a quantitation of the data in (B), showing the percentage of Cdc25 remaining in nuclei after 0 and 1 h. Values were normalized to coinjected GRP94 protein. (D) The N-terminal (aa. 1–322) fragment of wild-type or NES-null mutant Cdc25 proteins were fused to GST and coupled to glutathione–Sepharose beads. The pull-down assay was performed as in Figure 3A, and the bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-CRM1 and anti-14-3-3 ε antibody.

Intra-nuclear activation of Cdc25 S287A by Cdc2–cyclin B

In the above experiments, GVBD was preceded by nuclear accumulation and hyperphosphorylation of the S287A Cdc25, detected by an electrophoretic mobility shift upon SDS–PAGE (Figure 5B). A similar mobility shift of Cdc25 has been reported in response to phosphorylation by active Cdc2–cyclin B and by the kinase Pxl1 (Kumagai and Dunphy, 1992, 1996; Hoffmann et al., 1993; Izumi and Maller, 1995). In both cases, Cdc25 hyperphosphorylation correlates with an increase in its enzymatic activity. Since leptomycin B treatment also induces nuclear accumulation of Cdc2–cyclin B1, we wished to determine whether Cdc2–cyclin B might be responsible for the hyperphosphorylation of S287A Cdc25 in leptomycin-B-treated oocytes. Therefore, we repeated the wild-type and mutant Cdc25 injection experiments using oocytes which had been pre-incubated with both leptomycin B and the Cdc2 inhibitor, roscovitine (50 μM; Meijer et al., 1997). Roscovitine effectively eliminated the S287A Cdc25 mobility shift (and GVBD), consistent with a role for
Cdc2–cyclin B in the observed Cdc25 hyperphosphorylation (Figure 6A). At similar concentrations, roscovitine did not prevent Cdc25 hyperphosphorylation by kinases active in interphase egg extracts treated with the phosphatase inhibitor, microcystin (5 μM; Figure 6B). These egg extracts lack cyclins A and B (and hence lack active Cdc2), but are induced to enter a pseudomitotic state by incubation with microcystin and contain a variety of activated non-Cdc2 kinases including Plx1 (Izumi and Maller, 1995; Kumagai and Dunphy, 1996; Qian et al., 1998). Thus, the lack of inhibition of these non-Cdc2 kinases by roscovitine supports its reported specificity as an inhibitor and suggests that Cdc2–cyclin B was, indeed, responsible for phosphorylating and activating S287A Cdc25 in the oocyte. In aggregate, these data suggest that abrogating the Cdc25–14-3-3 interaction with the S287A mutation creates a more potent Cdc25 that triggers a feedback loop involving the mutual activation of Cdc25 and Cdc2/cyclin-B in the nuclei of leptomycin-B-treated oocytes.

The S287A mutant of Cdc25 is imported into nuclei more efficiently than the wild-type protein
What is the basis for the increased potency of S287A Cdc25? Since both Cdc2–cyclin B1 and Cdc25 gradually accumulate in oocyte nuclei upon inhibition of nuclear export with leptomycin B, one possibility is that the S287A mutation increases the nuclear import rate of Cdc25. Faster accumulation of the trace amount of injected Cdc25 in the nucleus (away from the countervailing inhibitory action of Myt1 on Cdc2–cyclin B) may allow it to trigger the activating feedback loop leading to GVBD. To compare the nuclear import rates of wild-type and S287A Cdc25 proteins, we repeated the cytoplasmic injection experiments, but used roscovitine to prevent GVBD and permit analysis of nuclear import (in leptomycin-B-treated oocytes, to prevent Cdc25 re-export). At various times after injection, oocytes were separated into cytoplasmic and nuclear fractions and resolved by SDS–PAGE. As shown in Figure 7, the S287A mutant protein accumulated in nuclei at a markedly faster rate than the wild-type protein. These data strongly suggest that S287 phosphorylation, and consequent 14-3-3 binding, reduces Cdc25 nuclear import in the oocyte.

How does 14-3-3 binding affect nuclear import of Cdc25? Scanning of the *Xenopus* Cdc25 sequence revealed an evolutionarily conserved, consensus bipartite basic NLS, KR X13 KRRR at amino acids 298–316. Consistent with its containing a classical NLS, the N-terminal 322 amino acids of Cdc25 bound to the nuclear import receptor for such sequences, importin-α/β (Figure 8A; Gorlich et al., 1994, 1995). Mutation of residues 313–315 of the candidate NLS to Ala severely impaired nuclear import of the mutant Cdc25 (Figure 8B) and greatly reduced binding to the importin-α/β heterodimer (Figure 8A). Consistent with its enhanced rate of nuclear import, the S287A Cdc25 mutant bound significantly better than the wild-type Cdc25 protein to the importin-α/β heterodimer (Figure 9A and B).
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Fig. 6. Cdc2–cyclin B is responsible for the hyperphosphorylation of Cdc25:S287A in leptomycin-B-treated oocyte nuclei. (A) Oocytes were incubated with either 200 nM leptomycin B in MB buffer for 2 h before injection, or with both 200 nM leptomycin B and 50 μM roscovitine. Forty nanoliters of 35S-labeled S287A protein translated in vitro was coinjected into oocyte cytoplasm with 14C-labeled BSA as control. Oocytes were dissected into cytoplasmic and nuclear fractions at the indicated times after injection and proteins were analyzed by SDS–PAGE followed by autoradiography. Oocytes not treated with roscovitine entered GVBD at 7 h, whereas those treated with roscovitine did not manifest GVBD even after incubation overnight. (B) Two microliters of 35S-labeled wild-type or S287A mutant Cdc25 protein was added to 20 μl of interphase Xenopus egg extract. These extracts were incubated with 5 μM microcystine, 50 μM roscovitine or 5 μM microcystin and 50 μM roscovitine at room temperature for 30 min in the presence of 2 mM ATP. The samples were subjected to SDS–PAGE and autoradiography.

When we injected NLS-null Cdc25 or S287A, NLS-null doubly mutant Cdc25 into oocyte nuclei to compare wild-type and S287A export rates under conditions where re-import could not occur, we did not observe any significant differences in their rates of nuclear export (Figure 9C). This demonstrates that the slight difference in the export rates of wild-type and S287A Cdc25 proteins shown in Figure 2C was due to faster re-import of exported S287A Cdc25. These data strongly suggest that 14-3-3 binding to Xenopus Cdc25 in oocytes exerts effects on nuclear shuttling by altering the rate of Cdc25 nuclear import, rather than export. Moreover, they indicate that 14-3-3 binding controls Cdc25 biological activity, at least in part, by inhibiting its entry into the nucleus.

Discussion

Binding of 14-3-3 to Cdc25 in G2-arrested oocytes

We have shown that Cdc25 in the G2-arrested oocyte can be found in a complex with 14-3-3 proteins, and that this complex is dissociated following progesterone treatment. In somatic cells, Cdc25 is phosphorylated in response to DNA damage and DNA-replication-induced checkpoint activation (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997). Recent studies have demonstrated that this phosphorylation (at residue S287 of Xenopus Cdc25) results in the formation of a 14-3-3–Cdc25 complex which is important for maintaining the checkpoint-induced G2 arrest (Peng et al., 1997; Kumagai et al., 1998b; Zeng et al., 1998). In Xenopus oocytes, S287 phosphorylation is also required for 14-3-3 binding. These parallels suggest that similar strategies are employed to maintain a G2 arrest in oocytes and somatic cells in response to different physiological stimuli. It will be interesting to determine whether other pathways functioning in the oocyte, such as regulation of Myt1 activity by rsk, also contribute to mitotic control in somatic cells.

14-3-3 binding selectively inhibits nuclear import of Cdc25 in oocytes

The mechanism whereby 14-3-3 binding functionally suppresses Cdc25 activity has been mysterious. We have found that Cdc25 accumulates almost exclusively in the cytoplasm of the oocyte as a result of a steady-state situation in which Cdc25 slowly enters the nucleus and is rapidly re-exported back to the cytoplasm. Our data demonstrate that the rate of Cdc25 nuclear import is greatly accelerated by mutation of S287 to non-phosphorylatable Ala. This suggests that S287 phosphorylation, and consequent 14-3-3 binding, significantly reduce Cdc25 nuclear import. We have identified a functional and evolutionarily conserved NLS in Cdc25 that lies adjacent to the site of 14-3-3 binding. Hence, 14-3-3 binding may sterically block access of Cdc25 to the nuclear import machinery.

Export of Cdc25 from oocyte nuclei was inhibited by leptomycin B, suggesting the involvement of the export factor CRM1. Indeed, we found that an N-terminal fragment of Cdc25 containing a putative NES sequence could bind to CRM1. Mutagenesis experiments demonstrated that this sequence was critical for both CRM1 binding and for nuclear export of Cdc25, and therefore constituted a functional NES. Unlike its dramatic effect on the nuclear import of Cdc25, mutation of S287 to Ala had no significant effect on the rate of Cdc25 nuclear export. This was confirmed in export assays in which re-import of Cdc25 was eliminated by mutation of the Cdc25 NLS. Thus, binding of 14-3-3 selectively reduces the rate of
Cdc25 nuclear import while leaving its rate of export unaffected, presumably resulting in more efficient exclusion of Cdc25 from the nucleus.

Very recently, Lopez-Girona et al. (1999) reported studies in the fission yeast *Schizosaccharomyces pombe* which also indicate that 14-3-3 binding leads to exclusion of Cdc25 from the nucleus. However, this similar outcome was proposed to arise from a distinct mechanism, in which 14-3-3 binding provided a portable NES for Cdc25 nuclear export. This is clearly not the case in *Xenopus* oocytes, because non-phosphorylatable Cdc25 incapable of binding 14-3-3 is fully competent for export mediated by the Cdc25-intrinsic NES. Furthermore, a mutant Cdc25 lacking a functional NES was unable to be exported despite its continued ability to bind 14-3-3, suggesting that in this system 14-3-3 binding is neither necessary nor sufficient for Cdc25 nuclear export. These apparent differences between fission yeast and *Xenopus* may reflect the fact that the NES we have identified in *Xenopus* Cdc25 does not appear to be evolutionarily conserved. Thus, different cells may use different mechanisms for achieving nuclear exclusion of Cdc25 in response to 14-3-3 binding.

14-3-3 binding to Cdc25 collaborates with CRM1-mediated nuclear export of unknown factors to maintain the G2 arrest in the oocyte

The importance of 14-3-3 binding for the suppression of Cdc25 biological activity was evident in experiments in which trace amounts of S287A Cdc25 injected into oocyte cytoplasm were able to induce GVBD, whereas similar amounts of wild-type Cdc25 were not. However, GVBD was only induced in oocytes treated with leptomycin B to inhibit CRM1-mediated nuclear export. A simple hypothesis to explain this requirement would be that oocyte maturation required retention of the imported Cdc25 in the nucleus. We tested this by injecting trace amounts of a doubly mutant Cdc25 lacking both a functional NES and the ability to bind 14-3-3. Although this protein was efficiently imported into and retained in oocyte nuclei, it did not promote GVBD unless oocytes were also treated with leptomycin B (data not shown). This suggests that factors other than Cdc25 must be retained in the nucleus to collaborate with the S287A Cdc25.

It has been reported that 14-3-3 binding to phosphorylated Cdc25 does not greatly affect its activity in vitro, producing a <2-fold reduction in activity (Peng et al., 1997; Kumagai et al., 1998b). However, even a slight increase in Cdc25 enzymatic activity resulting from the G2/M loss of 14-3-3 binding might be sufficient, after concentration in the nucleus, to activate a small amount...
of nuclear Cdc2–cyclin B; this would effectively set in motion a positive feedback loop. In the absence of bound 14-3-3 protein, Cdc25 may also be more susceptible to activation, either by Cdc2–cyclin B (in a feedback loop) or by other nuclear Cdc25-activating kinases. Reconstitution of the Cdc2–cyclin B–Cdc25 positive feedback loop with fully purified components will be required to distinguish between these possibilities. None the less, it is attractive to speculate that Cdc2–cyclin B is the factor which must be concentrated in the nucleus along with Cdc25 to promote passage through the G2/M transition. Once separated from the cytoplasmic Myt1 (and given the absence of nuclear Weel in oocytes), nuclear Cdc25 and Cdc2–cyclin B could very effectively activate each other through positive feedback mechanisms.

Materials and methods

Oocyte preparation, microinjection and subcellular fractionation

Stage VI oocytes of *Xenopus laevis* were prepared for microinjection, dissection and subcellular fractionation as described previously (Yang *et al*., 1998). Two injection controls for nuclear integrity were used: 14C-labeled bovine serum albumin (BSA; Amersham) and in vitro-translated 35S-labeled GRP94 (a protein that does not have an NES or NLS). At each timepoint, five to 10 oocytes were collected for subfractionation and analyzed by SDS–PAGE (National Diagnostics protigel), followed by autoradiography or Western blotting.

Preparation of Xenopus oocyte and egg extracts

Oocyte extracts were prepared as described previously (Shibuya *et al*., 1992). To induce GVBD, oocytes were treated with 5 μg/ml progesterone in modified Barth’s (MB) buffer (Swenson *et al*., 1989) overnight. Once GVBD was observed in the majority of oocytes, they were collected to make GVBD extracts; these extracts were prepared in mitotic egg extract buffer (Smythe and Newport, 1991) to preserve meiotic phosphorylations. Interphase egg extracts and mitotic extracts were prepared according to the protocols of Smythe and Newport (1991).

Coimmunoprecipitation experiments

Various extracts were incubated with the relevant sera at 4°C for 1 h. Protein A-Sepharose beads (Sigma, St Louis, MO) were then washed with extract buffer and incubated with the above extracts at 4°C for 2 h. Beads were pelleted and washed five times with the relevant extract buffer. Bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting.

In vitro translation

*Xenopus* Cdc25 wild-type, S287A, NLS-null and NES-null mutants were subcloned downstream of an SP6 promoter in the vector SP64T. 35S-labeled proteins were produced using the SP6-coupled TNT reticulocyte system (Promega) according to the manufacturer’s instructions.

Construction of the GST-N-terminal and C-terminal Cdc25 fusion proteins and the S287A Cdc25 mutant

To construct the N-terminal fragment of Cdc25, a stop codon was inserted from amino acid 322 towards the N-terminus of Cdc25 in pGexKG. The Erase-a-Base system (Promega) was used to generate a deletion series from amino acid 322 towards the N-terminus of Cdc25 in pGexKG. The DNA encoding the N-terminal fragment of Cdc25 in pGexKG was cut at its 3’ end with *Xho*I to generate a 5’ overhang for Exonuclease III digestion. It was also digested with SacI to generate an adjacent 3’ overhang to protect the plasmid vector from Exonuclease III digestion. Exonuclease III deletion, ligation and transformation protocols were as

![Fig. 9. The Cdc25:S287A mutant binds significantly better than the wild-type protein to importin-αβ, and import-defective variants of these proteins export from nuclei at a similar rate. (A) *Xenopus* importin-α protein was fused to GST and coupled to glutathione-Sepharose beads. Twenty microliters of this resin or control GST resin was incubated in 100 μl of interphase egg extract with 10 μl 35S-labeled wild-type or S287A Cdc25 protein for 1 h. The beads were then pelleted and washed five times with egg lysis buffer. The bead-bound proteins were analyzed by SDS–PAGE followed by autoradiography. (B) The bar graph represents a quantitation of the data in (A), comparing the amounts of Cdc25:WT or Cdc25:S287A bound to importin-αβ (importin-α beads will associate with importin-β in the extract). (C) Cdc25:NLS-null or S287A:NLS-null protein was translated in reticulocyte lysates in the presence of [35S] methionine (extract). (D) The graph represents a quantitation of the data in (C), showing the percentage of Cdc25 remaining in nuclei after 0, 30 and 60 min.](image-url)

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described by the manufacturer. Fifty clones were induced for GST-fusion protein expression. Based on their sizes, five clones were selected for binding assays.

Site-directed mutagenesis

For construction of the NES-null mutant Cdc25, PCR-based mutagenesis was used to mutate both Leu53 and Val55 to Ala. Wild-type Xenopus Cdc25 cDNA in the pSP64T vector was used as the template. The 5′ oligonucleotide encoding the N-terminus of Cdc25 was 5′-ATATAGTG-AAGCCATGGCAGAGAGTGACACAAT-3′, where an NcoI site was inserted before the start codon. The 3′ oligonucleotide encoding the C-terminus of Cdc25 was 5′-GCCGCGCGCCCTGAGATTAAAGGCTT-CATCAGGCG-3′, where an XhoI site was inserted after the stop codon. The mutagenic PCR primers were 5′-TGTACCTGTTGACTGCCGCTGACCTGAGTGAGGACTG-3′ and its reverse primer. Using the 5′ primer along with one mutagenic primer, we produced a PCR fragment extending from the 5′ end (encoding the extreme N-terminus) to the site of mutation. We then generated a second PCR product using the C-terminal primer and the second mutagenic primer, producing a DNA fragment extending from the mutation site to the C-terminal end. A full-length mutant clone was generated with an oligonucleotide 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 construction of the NLS-null mutant Cdc25, the same strategy was used to mutate Lys313, Arg314 and Arg315 to Ala in order to destroy the consensus bipartite NLS. The PCR primers containing mutations were 5′-GAACACCTGTCAGAGTGGCCGCGGCACGTA-GTACCCAGCCGCC-3′ and its reverse primer. All mutations were confirmed by DNA sequencing.

Expression and purification of recombinant GST-fusion proteins

All constructs were expressed in Topp 3 Escherichia coli (Stratagene). To increase solubility of recombinant proteins, bacteria were grown to OD 0.5 at 37°C and then shifted to 18°C. Isopropyl-β-D-thiogalactoside (0.4 mM) was added to induce protein expression at 18°C overnight. Bacteria were pelleted and resuspended in lysis buffer [50 mM Tris7.5, 10 mM KCl, 1 mM EDTA, 1 mM diithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. 1% Triton X-100 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 HEPES 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 HEPES, pH 8.0, 300 mM NaCl and 1 mM DTT) and kept in storage buffer (10 mM HEPES pH 8.0, 50% glycerol, 1 mM DTT) at −20°C.

Pull-down experiments

GST fusion proteins were coupled to glutathione-Sepharose beads and washed with the appropriate extract buffer. The beads were then incubated with either different extracts or with extracts containing 35S-labeled proteins at 4°C for 1–2 h. The beads were washed five times with the proper extract buffer and the binding proteins were resolved by SDS–PAGE followed by immunoblotting or autoradiography.

Histone H1 kinase assay of single oocytes

Single oocytes were thawed in 20 μl of oocyte lysis buffer (20 mM HEPES 7.5, 80 mM β-glycerophosphate, 15 mM MgCl2, 20 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 10 μg/ml aprotinin/leupeptin, 1 mM PMSF, 1 mM DTT) and lysed by pipetting. The sample was then microfuged at 4°C for 5 min and the supernatant was collected. To start the kinase reaction, 10 μl of the supernatant was incubated with 10 μl kinase buffer (20 mM HEPES 7.3, 10 mM EGTA, 20 mM MgCl2, 10 μM protein kinase inhibitor (PKI), 0.2 mg/ml histone H1, 0.2 mM ATP and 0.5 μCi/μl [γ-32P]ATP) at room temperature for 10 min. The reaction was stopped by addition of 20 μl of 2× SDS sample buffer and subjected to SDS–PAGE and autoradiography.

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References

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