Initial Triggering of M-Phase in Starfish Oocytes: A Possible Novel Component of Maturation-promoting Factor Besides cdc2 Kinase

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Abstract. G2-phase–arrested immature starfish oocytes contain inactive cdc2 kinase and cdc25 phosphatase, and an inactivator for cdc2 kinase. In this system, we have studied how the regulatory balance is tipped toward the initial activation of cdc2 kinase. During the hormone-dependent period (Guerrier, P., and M. Doree. 1975. *Dev. Biol.* 47:341–348), $p34^{cdc2}$ and cdc25 protein are already converted, though not fully, to active forms, whereas the inactivators for cdc2 kinase and cdc25 phosphatase are able to exhibit their activities if the hormone were removed. We produced "triggered oocytes," in which due to a neutralizing anti-cdc25 antibody, the activation of cdc2 kinase is prevented but cdc25 protein is phosphorylated slightly after the maturation-inducing hormonal stimulus. In contrast to control immature oocytes, in triggered oocytes the injected cdc2 kinase is not inactivated, and accordingly the level of cdc2 kinase activity required for meiosis reinitiation is much less. These results imply the presence of a cdc2 kinase activity-independent process(es) that suppresses the inactivator for cdc2 kinase and initially phosphorylates cdc25 protein, although this process is reversible during the initial activation of cdc2 kinase. At the most initial triggering of M-phase, the cdc2 kinase activity-independent process might trip the switch leading to the initial activation of cdc2 kinase. Thereafter, in parallel, the cdc2 kinase-dependent feedback loops described by others may cause further increase in cdc2 kinase activity. We propose that a putative suppressor, which downregulates the inactivator for cdc2 kinase independently of nuclear components, might be a previously unrecognized component of maturation-promoting factor.

THE cell cycle in primary oocytes arrests at the prophase of the first meiosis which corresponds to the G2/M-phase border, and hence the presence of a G2 checkpoint is postulated specifically in oocyte meiotic cell cycle (see Murray and Hunt, 1993). The release of oocytes from the G2 checkpoint is finally performed by a maturation-promoting factor (MPF).[1] MPF was originally identified functionally as a cytoplasmic activity which reinitiates the first meiosis in immature oocytes in the absence of the normal maturation-inducing hormonal stimulus (Masui and Markert, 1971; Kishimoto and Kanatani, 1976). The M-phase–inducing activity contained in MPF is now established to be ascribed to cdc2 kinase, the active form of the cyclin B/$p34^{cdc2}$ complex, which governs both entry into and exit from M-phase in all eukaryotic cells (for reviews see Hunt, 1989; Doree, 1990; Nurse, 1990; Maller, 1991; Kishimoto, 1994). The protein kinase activity of the cyclin B/$p34^{cdc2}$ complex is controlled by phosphorylation/dephosphorylation of three major residues, Thr14, Tyr15, and Thr161, in the $p34^{cdc2}$ subunit that is associated with newly synthesized cyclin B protein (for reviews see Coleman and Dunphy, 1994; King et al., 1994). Phosphorylation of Thr161 residue by a cdk-activating kinase (CAK), the cyclin H/cdk7 complex, is indispensable for the cdc2 kinase activity (for reviews see Solomon, 1994; Morgan, 1995). Phosphorylation of both Thr14 and Tyr15 residues suppresses the cdc2 kinase activity, whereas their dephosphorylation results in the activation (for reviews see Coleman and Dunphy, 1994; Morgan, 1995), indicating that the balance between the activities of phosphorylation and dephosphorylation defines the onset of M-phase.

A protein kinase that is possibly responsible for phosphorylation of both Thr14 and Tyr15 has been recently demonstrated (Atherton-Fessler et al., 1994; Kornbluth et al., 1994), and wee1/mik1 kinase is known to phosphorylate at least the Tyr15 residue (Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993, 1995; Mueller et al., 1995; Watanabe et al., 1995). A cdc25 phosphatase dephosphorylates both the Thr14 and Tyr15 residues (Gautier et al., 1991; Kumagai and Dunphy, 1991; Straus-

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1. *Abbreviations used in this paper*: a.u., arbitrary unit; GVBD, germinal vesicle breakdown; MPF, maturation-promoting factor; 1-MeAde, 1-methyladenine.

feld et al., 1991; Izumi et al., 1992; Sebastian et al., 1993; for review see Millar and Russell, 1992). The activity of cdc25 phosphatase is increased by its phosphorylation with the active cdc2 kinase, indicating that cdc25 phosphatase and cdc2 kinase constitute a positive feedback loop for the amplification of cdc2 kinase activity (Kumagai and Dunphy, 1992; Hoffmann et al., 1993; Izumi and Maller, 1993). Similarly, the Thr14/Tyr15 kinase is downregulated at M-phase (Atherton-Fessler et al., 1994; Kornbluth et al., 1994), and also wee1/mik1 kinase is negatively regulated by the phosphorylation with the mitotic inducer nim1/cdr1 kinase, the active cdc2 kinase and/or an unknown kinase (Coleman et al., 1993; Parker et al., 1993; Tang et al., 1993; Wu and Russell, 1993; McGowan and Russell, 1995; Mueller et al., 1995; Watanabe et al., 1995; for review see Coleman and Dunphy, 1994).

In spite of these individual studies in vitro, however, it is still unclear how these regulatory components for the cyclin B/p34^{cdc2} complex are coordinated in vivo, in particular how the balance between the phosphatase for activation and the kinase for inactivation is tipped to initiate the feedback loops toward cdc2 kinase activation. In this context, Kuang et al. (1991) suggested the presence of multiple forms of MPF in *Xenopus* eggs, and it is not yet exactly clear whether cdc2 kinase is the only responsible component of the original cytoplasmic MPF. No report has yet compared precisely the M-phase–inducing activity in oocytes between the original cytoplasmic MPF and the purified cdc2 kinase (for example see Gautier et al., 1988; Labbe et al., 1989). In fact, the injected cdc2 kinase is inactivated in immature starfish oocytes (Picard et al., 1991), while the cytoplasmic MPF activity that is detectable upon injection into immature starfish oocytes requires the participation of nuclear components (Kishimoto et al., 1981; Picard and Doree, 1984; Picard et al., 1991). It would be rather anticipated that the switch that leads to the initial activation of cdc2 kinase in vivo might be tripped by other factor(s) than cdc2 kinase, and that the factor(s) might constitute the original cytoplasmic MPF besides cdc2 kinase.

Fully grown immature oocytes of starfish, which are arrested at G2-phase, already contain an inactive form of cyclin B/p34^{cdc2} complex, in which all of the Thr14, Tyr15, and Thr161 residues of p34^{cdc2} seem to be phosphorylated (see Strausfeld et al., 1991; Ookata et al., 1992). In this study, we produced triggered starfish oocytes in which the activation of cdc2 kinase after the maturation-inducing hormonal stimulus was prevented by a neutralizing antibody against cdc25 phosphatase but nevertheless, cdc25 protein was slightly phosphorylated. To study the initial process for cdc2 kinase activation, we rapidly purified the active cdc2 kinase, and compared its fate upon injection in control immature oocytes and in triggered oocytes. The results indicate that independently of the cdc2 kinase activity and the nuclear components, an inactivator for cdc2 kinase, which is present in immature oocytes and appears to be related to the Thr14/Tyr15 kinase and/or wee1 kinase, was suppressed at the release of oocytes from the G2 checkpoint. Accordingly, the level of cdc2 kinase activity that was required for meiosis reinitiation was much less in triggered oocytes than in control immature oocytes. We propose that besides cdc2 kinase, a putative suppressor for the inactivator of cdc2 kinase is a previously unrecognized component of the original cytoplasmic MPF. Candidates for the suppressor activity and for the initial activator to cdc25 phosphatase are discussed.

Materials and Methods

Starfish Oocytes and Their Extracts

Immature oocytes or maturing oocytes at the first meiotic metaphase after 1-methyladenine (1-MeAde) treatment were prepared from starfish, *Asterina pectinifera* as described previously (Kishimoto and Kondo, 1986). Starfish oocyte extracts were obtained according to Labbe et al. (1989, 1991). Briefly, 10 ml of packed oocytes was Teflon/glass homogenized with 20 ml of a buffer A containing 80 mM Na-β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$ and 1 mM DTT (pH 7.3), supplemented with 100 mM KCl, 100 mM sucrose, 0.3 mM PMSF, and 20 μg/ml leupeptin. The mixture was centrifuged at 14,000 *g* for 15 min at 4°C, followed by recentrifugation of the supernatant at 140,000 *g* for 40 min to recover high speed extracts.

Enucleated oocytes were prepared from immature oocytes as described previously (Kishimoto et al., 1981; Kishimoto, 1986).

Purification of Cyclin B–Dependent cdc2 Kinase with p13^{suc1}-Affinity Chromatography

Affinity chromatography with p13^{suc1}-conjugated Sepharose 4B was performed as previously described (Kusubata et al., 1992), with slight modifications. Briefly, the high speed extracts (20 ml, 400 mg protein) containing cdc2 kinase from maturing starfish oocytes were recleared by centrifugation at 140,000 *g* for 40 min after thawing, and then preincubated with control Sepharose 4B beads (3 ml) for 1 h at 4°C with gentle stirring. The mixture was centrifuged at low speed, and the supernatant was batch-loaded on 3 ml of p13^{suc1}-Sepharose 4B equilibrated with buffer B, in which buffer A was supplemented with 0.01% Brij35. After a 60-min incubation at 4°C under gentle agitation, the p13^{suc1}-beads were packed into a chromatographic column. The column was washed with 15 ml of buffer B supplemented with 500 mM NaCl, followed by 15 ml of buffer B, 15 ml of buffer B supplemented with 30% ethylene glycol, and finally starfish cdc2 kinase was eluted with 15 ml of buffer B containing both 500 mM NaCl and 50% ethylene glycol. Eluted fractions were dialyzed against buffer A supplemented with 10% sucrose and 0.1% NP-40 instead of 0.01% Brij35. For microinjection into oocytes, the dialyzed preparation was concentrated with Ultrafree C3TK (Millipore, Bedford, MA) in the presence of BSA at a final concentration of 1 mg/ml. Inactive form of the cyclin B/p34^{cdc2} complex was purified from immature starfish oocytes with the same procedure.

For human cdc2 kinase, HeLa cells were synchronized at M-phase with nocodazole after a thymidine block. The cells were homogenized with a low salt buffer containing 50 mM NaF, 50 mM Na-β-glycerophosphate, 1 mM $MgCl_2$, 1 mM EGTA and 50 mM imidazole, pH 7.0, followed by centrifugation at 100,000 *g* for 1 h. Human cdc2 kinase was extracted with buffer B containing 400 mM NaCl from the precipitate (Hisanaga et al., 1995), and was p13^{suc1}-affinity purified according to Kusubata et al. (1992).

Protein concentration was determined by bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard. SDS-PAGE was carried out by the method of Laemmli (1970) with 8% or 12% acrylamide gel stained with silver or Coomassie blue.

Microinjection and Histone H1 Kinase Assay

Microinjection or transfer of MPF-containing cytoplasm into starfish oocytes was performed as described previously (Kishimoto and Kanatani, 1976; Kishimoto, 1986). GVBD in recipient oocytes was inspected within 2 h after the injection. For SDS-PAGE, 10 recipient oocytes were recovered in 2 μl of seawater at an appropriate time after the microinjection, added with 10 μl of 2× concentrated SDS sample buffer, and immediately frozen in liquid N_2. After boiling, the whole of each sample was applied to each lane of SDS-PAGE. When recipient oocytes were recovered just after the injection, microinjection was performed in seawater at 4°C, and pairs of recipient oocytes were immediately transferred to an excess amount of cold seawater. Then, 10 recipient oocytes were recovered finally in 2 μl of cold seawater.

For histone H1 kinase assay of microinjected oocytes, 10 recipient oocytes were rapidly washed in an excess amount of buffer A, recovered in 2

μl of buffer A, and immediately frozen in liquid N_2. After thawing, 8 μl of a mixture containing 0.125 mM [γ-^{32}P]ATP (0.625 mCi), 6.25 mM $MgCl_2$, and 1.25 mg/ml histone H1 (Boehringer Mannheim Corp., Indianapolis, IN) was added immediately, and the mixture was incubated for 20 min at 20°C. Kinase reactions were terminated by spotting 2.5-μl aliquots onto Whatman P81 phosphocellulose paper, and after 15 min, the filters were washed five times (5 min each) in a 200-ml solution of 1% phosphoric acid. The wet filters were transferred into scintillation vials, 5 ml of ACSII (Amersham Corp., Arlington Heights, IL) scintillation fluid was added and the samples were counted in an Aloka scintillation counter. Finally, the histone H1 kinase activity contained in an oocyte was estimated by assuming that the volume of an oocyte was 4 nl, i.e., that cytoplasm was diluted 250-fold in 10 μl of kinase reaction mixture. For assaying cdc2 kinase preparations, the samples were diluted 50-fold with buffer A, and then the histone H1 kinase activity was measured in 2 μl of sample as described above. In Figs. 3 *C*, 4, and 6, the histone H1 kinase activity is represented in arbitrary unit (a.u.) to facilitate the comparison among these figures, where 100 a.u. corresponds to the histone H1 kinase activity contained in a control maturing oocyte at the first meiotic metaphase after 1-MeAde addition.

Antibodies and Immunological Methods

A cDNA of starfish homolog of *S. pombe* cdc25 was isolated, and anti-starfish cdc25 antiserum was raised in rabbits by subcutaneous injection of bacterially expressed fragment coding the COOH-terminal 153 amino acids of starfish cdc25. The anti-starfish cdc25 antibody was further affinity purified using nitrocellulose strips onto which the bacterially produced starfish cdc25 fragment was transferred (see Ookata et al., 1992). The specificity of the anti-cdc25 antibody was confirmed by its preincubation with the bacterially produced starfish cdc25 peptide which completely eliminated the reaction with the 90-kD band (see Fig. 1 *D*, lane *1*) in lysate of metaphase oocytes (data not shown). For microinjection, the purified anti-starfish cdc25 antibody was concentrated in Tris-buffered saline (TBS), pH 7.5 with Ultrafree C3TK as described above.

A cDNA of starfish cdc2 was isolated. An anti-starfish cdc2 antiserum was raised in rabbits against a synthetic peptide corresponding to the COOH-terminal 14 amino acids of starfish cdc2 protein (DFEGGTVLPTRLGQ), and further affinity purified. Anti-starfish cyclin B antibody was raised in rabbits against bacterially produced starfish cyclin B as described previously (Ookata et al., 1992). Anti-phosphotyrosine antibody and anti-human cdc2 antibody against its COOH-terminal peptide were generous gifts from Drs. G. Peaucellier (Roscoff, France) and F. Matsumura (Rutgers University, Piscataway, NJ), respectively. Immunoblotting and immunoprecipitation were performed essentially as described previously (Ookata et al., 1992). For the second antibody of immunoblotting, alkaline phosphatase–conjugated swine anti–rabbit IgG (DAKO) was used at a dilution of 1:400.

Purification of Starfish cdc25 Phosphatase and In Vitro Activation of cdc2 Kinase by cdc25 Phosphatase

Anti-starfish cdc25 antibody was cross-linked with protein A–Sepharose CL4B (Pharmacia LKB Biotechnology, Piscataway, NJ) by DSS (2, 2-dimethyl-2-silapentane-5-sulfonate; Pierce) at 5 mg IgG/ml beads according to the manufacturer's instructions. 15 ml of the flowthrough fractions from p13^{suc1}-affinity column after the application of high speed extracts of maturing starfish oocytes was mixed with 1 ml of the anti-cdc25 antibody beads for 1 h at 4°C, and then the suspension was packed into a column. After washing the column with 10 ml of a buffer containing 500 mM NaCl, 50 mM Tris (pH 7.5) and 1 mM DTT, starfish cdc25 protein was eluted from its antibody-beads with 4.5 M $MgCl_2$ solution, pH 7.5. 3 ml of pooled fractions were desalted with PD-10 column (Pharmacia LKB Biotechnology) equilibrated with a buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 1 mM DTT, and 0.1% NP-40. The cdc25 fractions were concentrated with Ultrafree C3TK as described above.

To examine the effect of anti-cdc25 antibody on the in vitro activation of the cyclin B/p34^{cdc2} complex by cdc25 phosphatase, 1.5 μl of the purified active cdc25 phosphatase at a concentration of 0.05 mg/ml was preincubated for 30 min at 2°C with 0.75 μl of either the anti-cdc25 antibody or control IgG at a concentration of 1 mg/ml, and then each mixture was added with 0.75 μl of the purified inactive form of cyclin B/p34^{cdc2} complex at a concentration of 0.05 mg/ml. After 60 min incubation at 25°C, 2 μl of the incubation mixture was processed for the histone H1 kinase as-

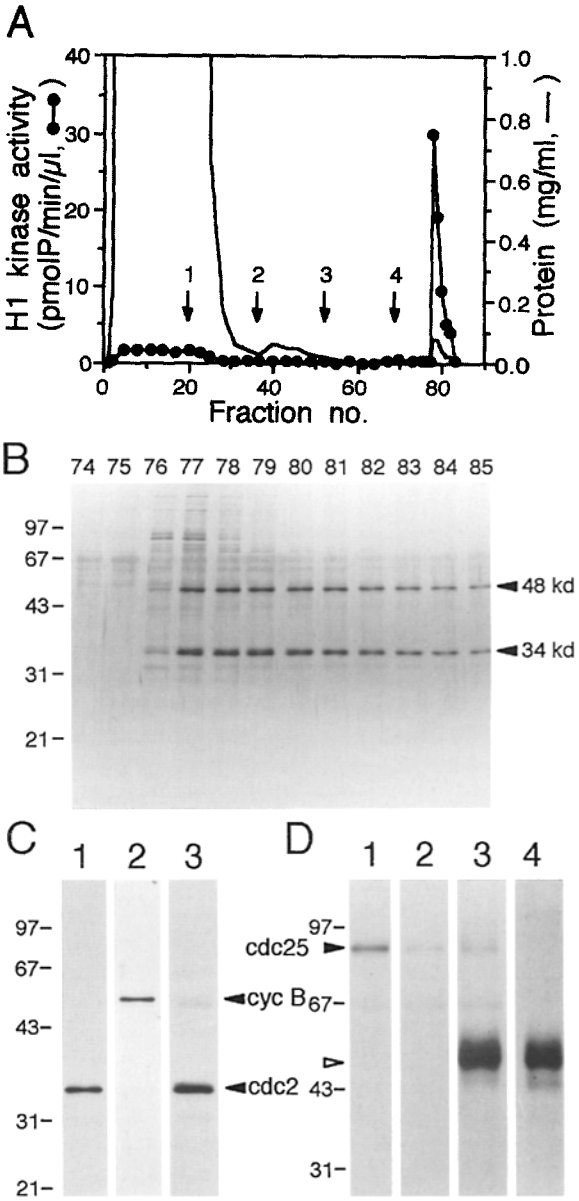

Figure 1. Purification of cyclin B–dependent cdc2 kinase with p13^{suc1}-affinity column from starfish oocytes. (*A*) Elution profile of starfish cdc2 kinase from p13^{suc1}-Sepharose. After preincubation with control Sepharose beads, high speed extracts from starfish oocytes at the first meiotic metaphase were mixed with p13^{suc1}-Sepharose equilibrated with buffer B, and then the suspension was packed into a column. Thereafter, the column (bed volume, 3 ml) was washed with buffer B supplemented with 500 mM NaCl (*arrow 1*), none (*arrow 2*), 30% ethylene glycol (*arrow 3*), and finally both 500 mM NaCl and 50% ethylene glycol (*arrow 4*). (*B*) Silver staining of SDS-PAGE of fractions eluted by buffer B containing both 500 mM NaCl and 50% ethylene glycol. (*C*) Western blots of the peak fraction of No.77 in (*B*) with the anti-starfish cdc2 antibody (lane *1*) and with the anti-starfish cyclin B antibody (lane *2*), and of anti-cyclin B immunoprecipitate of the peak fraction with the anti-cdc2 antibody (lane *3*). (*D*) Western blots with the anti-starfish cdc25 antibody of high speed extracts from starfish oocytes at the first meiotic metaphase (lane *1*), p13^{suc1}-Sepharose adsorbed fraction (lane *2*), anti-cyclin B immunoprecipitate of the high speed extracts (lane *3*), and anti-cyclin B immunoprecipitate of the peak fraction eluted with both NaCl and ethylene glycol (lane *4*). Open arrowhead indicates rabbit IgG.

say as described above. In Fig. 3 *A*, one arbitrary unit (a.u.) of histone H1 kinase activity corresponds to that at the beginning of incubation.

Results

Rapid Purification of suc1 and cdc25-free Cyclin B–dependent cdc2 Kinase

In the present study, we used the purified cdc2 kinase to dissect the initial process for cdc2 kinase activation. Since previous studies suggest various effects of suc1 and cdc25 proteins on cdc2 kinase activity (see Gautier et al., 1991; Kusubata et al., 1992; Zheng and Ruderman, 1993), we first modified the reported methods (Labbe et al., 1989, 1991; Kusubata et al., 1992) and purified cdc2 kinase free of these proteins from maturing starfish oocytes. High speed extracts of maturing starfish oocytes were precleared of proteins which bound nonspecifically to Sepharose 4B beads, and then adsorbed to $p13^{suc1}$-Sepharose affinity gels (Fig. 1 *A*). Buffers containing either 500 mM NaCl or 30% ethylene glycol alone were effective for washing the $p13^{suc1}$-Sepharose column, and histone H1 kinase activity was almost specifically eluted by the buffer containing both 500 mM NaCl and 50% ethylene glycol. Single step affinity chromatography yielded 500–1,000-fold purification with ~50% recovery of the histone H1 kinase activity.

In peak fractions eluted from $p13^{suc1}$-affinity column, the major proteins were 48 and 34 kD, each of which was identified as cyclin B and cdc2 protein by Western blots, respectively (Fig. 1, *B* and *C*). Immunoprecipitation with anti-cyclin B antibodies confirmed the association of $p34^{cdc2}$ with cyclin B. The anti-cyclin B immunoprecipitate recovered almost all the histone H1 kinase activity which was contained in peak fractions, as the cyclin A/$p34^{cdc2}$ kinase was almost undetectable during first meiotic cycle in starfish oocytes (Okano, T., and T. Kishimoto, manuscript in preparation).

Affinity-purified anti-starfish cdc25 antibody recognized a single band in the lysate of starfish oocytes at the first meiotic metaphase with an apparent molecular size of 90 kD (Fig. 1 *D*, lane *1*). Starfish cdc25 protein was detectable both in the anti-cyclin B immunoprecipitate from the high speed extracts of maturing oocytes and in the $p13^{suc1}$ beads-adsorbed fraction before the washing with 0.5 M NaCl, but not in the anti-cyclin B immunoprecipitate of the cdc2 kinase preparation which was eluted from $p13^{suc1}$-affinity column (Fig. 1 *D*, lanes *2–4*). Although at least a part of the cyclin B/$p34^{cdc2}$ complex was associated with cdc25 protein in lysates of maturing starfish oocytes as reported by Jessus and Beach (1992), cdc25 protein was released from the complex during the washing of the $p13^{suc1}$-gels. In contrast to Labbe et al. (1989, 1991), $p13^{suc1}$ was not contained in the present elution buffer for $p13^{suc1}$-affinity column. We conclude that the present preparation of purified cdc2 kinase was free of both cdc25 and suc1 proteins.

Reversible Activation of Cyclin B–dependent cdc2 Kinase and cdc25 Phosphatase during Hormone-dependent Period

In immature starfish oocytes, a maturation-inducing hormone, 1-methyladenine (1-MeAde), causes the activation of cyclin B/$p34^{cdc2}$ complex and induces GVBD within 30 min without the requirement for new protein synthesis. To examine the process leading to the initial activation of cdc2 kinase, we took advantage of the so-called "hormone-dependent period" (Guerrier and Doree, 1975), which corresponds to a minimum required duration of several minutes for 1-MeAde to be present in seawater to induce GVBD: if 1-MeAde is removed before the end of hormone-dependent period, oocytes fail to undergo GVBD. We monitored the modification of both cdc25 and cdc2 proteins after 1-MeAde addition, and followed the effect of the removal of 1-MeAde during the hormone-dependent period on their modification.

When 1-MeAde was continuously present and induced GVBD, the electrophoretic mobility of cdc25 protein was retarded gradually from 80 kD in immature oocytes to 90

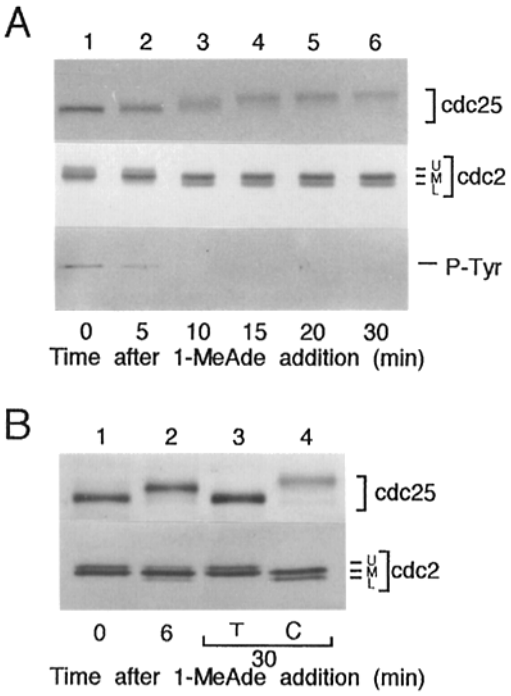

Figure 2. Reversible modification of cdc2 and cdc25 proteins during the hormone-dependent period of starfish oocytes. (*A*) Mobility shift of cdc25 and cdc2 proteins in starfish oocytes after meiosis reinitiation by 1-MeAde. Starfish oocytes were collected various times after 1-MeAde addition (indicated below by min), and then cdc25 protein (*upper panel*), cdc2 protein (*middle panel*), and phosphorylated Tyr-residue at 34 kD were monitored by Western blots. The middle band of cdc2, cdc2-M, is not associated with cyclin B in the G2-phase arrested oocytes and metaphase oocytes (see Fig. 4 in Ookata et al., 1992). (*B*) Mobility shift of cdc25 and cdc2 proteins is reversed by the removal of 1-MeAde during the hormone-dependent period. After the addition of 1 μM 1-MeAde to immature starfish oocytes, 1-MeAde was removed at 6 min, i.e., before the end of the hormone-dependent period, by immediate and extensive dilution with seawater, resulting in no induction of GVBD (*T*, transient; lane *3*). As a control, 1-MeAde was continuously present to induce GVBD at 20 min (*C*, continuous; lane *4*). Modifications in cdc25 (*upper panel*) and cdc2 (*lower panel*) proteins were monitored by the electrophoretic mobility shift visualized by Western blots in intact immature oocytes (0 min, lane *1*), and oocytes at 6 min (lane *2*) or at 30 min (lanes *3* and *4*) after 1-MeAde addition.

kD in oocytes that had undergone GVBD (Fig. 2 *A*, *upper panel*). This gradual mobility shift appears to reflect the levels of phosphorylation and activation of cdc25 phosphatase which were reported in progesterone-treated *Xenopus* oocytes (Izumi et al., 1992). In accordance with the notion, coincidentally with the mobility shift of cdc25 protein, the slowest migrating form of starfish cdc2 protein, cdc2-U, which represents the inactive form of cyclin B-associated $p34^{cdc2}$ with presumably phosphorylated Thr14 and Tyr15 residues (see Strausfeld et al., 1991; Fig. 4 in Ookata et al., 1992), disappeared, and the fastest migrating form of cdc2 protein, cdc2-L, which represents the active form with presumably dephosphorylated Thr14 and Tyr15 residues, appeared reciprocally (Fig. 2 *A*, *middle panel*). In accordance with this shift, the phosphorylated form of Tyr residue became undetectable at 34 kD in oocyte lysates (Fig. 2 *A*, *lower panel*). As shown clearly in Fig. 2 *B* (lane *2*; compare with lane *4*), 6 min after the addition of 1-MeAde to immature oocytes, the active form of cdc2 protein, the cdc2-L was already detectable, and the shift-up of cdc25 protein, though not full, occurred coincidentally. At this time point, the activity of histone H1 kinase was already in the process of increase (see Fig. 4 in Ookata et al., 1992).

In contrast to continuous treatment, if 1-MeAde was removed at 6 min, GVBD failed to occur and levels of both cdc2 and cdc25 proteins returned within 30 min to the initial states that were seen in immature oocytes (Fig. 2 *B*, lane *3*). These observations indicate that potent inactivators for cdc2 kinase and cdc25 phosphatase, i.e., a kinase(s) for cdc2 protein, presumably a Thr14/Tyr15 kinase(s) and/or wee1-like kinase, and a phosphatase for cdc25 protein, exhibit their activities even after the partial activation of cdc2 kinase and cdc25 phosphatase at least during the hormone-dependent period if 1-MeAde was removed. In this context, Picard et al. (1991) reported that the injected cdc2 kinase is inactivated in immature starfish oocytes in which the intrinsic cdc2 kinase is not yet activated (see also Fig. 4).

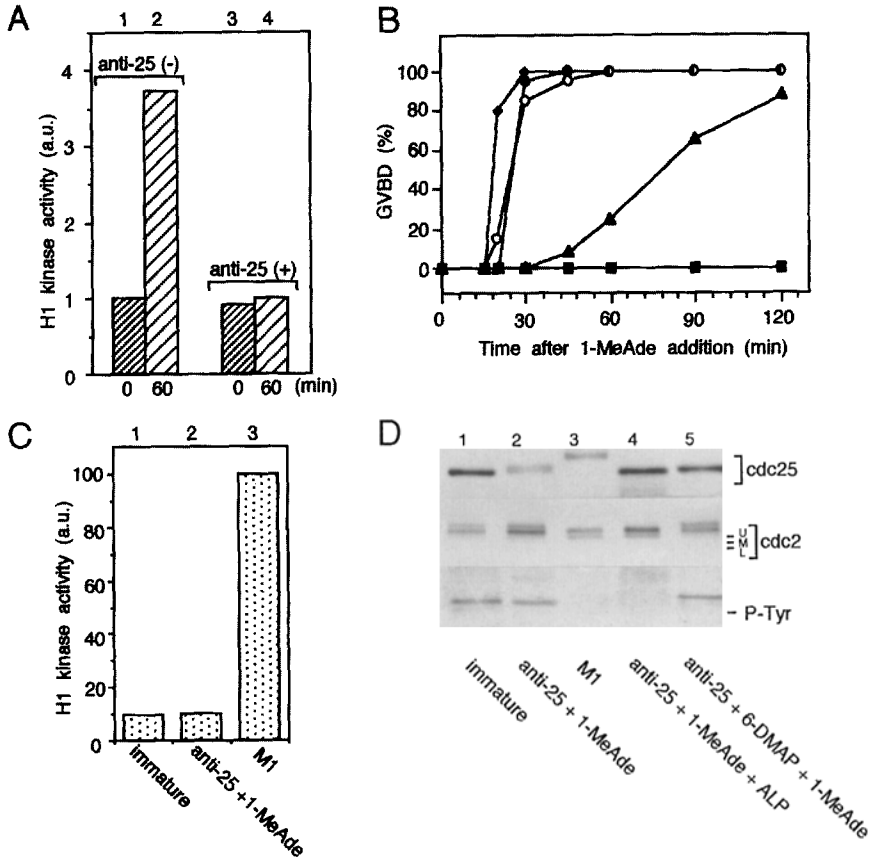

Figure 3. The anti-starfish cdc25 antibody neutralizes the cdc25 phosphatase activity and prevents the activation of cdc2 kinase. (*A*) The anti-starfish cdc25 antibody prevented the in vitro activation of the cyclin B/$p34^{cdc2}$ complex by cdc25 phosphatase. Inactive form of cyclin B/$p34^{cdc2}$ complex was purified from immature starfish oocytes with $p13^{suc1}$-affinity column. Active starfish cdc25 phosphatase was purified with anti-cdc25 antibody-conjugated protein A–Sepharose from starfish oocytes at the first meiotic metaphase. The cdc25 phosphatase was incubated for 30 min at 2°C in the presence (bars *3* and *4*) or the absence (bars *1* and *2*) of the anti-cdc25 antibody. Thereafter, the activity of the cdc25 phosphatase to activate the inactive form of cyclin B/$p34^{cdc2}$ complex was estimated from the increase in histone H1 kinase activity after the 60-min incubation at 25°C. (*B*) The anti-cdc25 antibody prevents 1-MeAde–induced starfish oocyte maturation. The affinity-purified anti-starfish cdc25 antibody was concentrated, and various amounts of it (0 pg, ◆; 1 pg, ●; 5 pg, ▲; 20 pg, ■) was injected into 10 immature starfish oocytes each. As a control, 20 pg of IgG (○), which was equivalent to the largest amount of the purified antibody, was injected. 30 min after the microinjection, oocytes were treated with 1 μM 1-MeAde, and then GVBD was inspected at the indicated times. (*C*) Histone H1 kinase is not activated after 1-MeAde treatment in oocytes injected previously with the anti-starfish cdc25 antibody. Histone H1 kinase activity was measured in intact immature starfish oocytes (bar *1*), oocytes injected with 20 pg of the anti-cdc25 antibody followed by 1-MeAde treatment for 30 min (bar *2*; no GVBD), and control maturing oocytes at the first meiotic metaphase (bar *3*, M1). (*D*) Cdc25 protein, but not cdc2 protein, is slightly modified after 1-MeAde treatment in oocytes injected previously with the anti-starfish cdc25 antibody. Western blots of control immature starfish oocytes (lane *1*), oocytes injected with 20 pg of the anti-cdc25 antibody followed by 1-MeAde treatment for 30 min (lane *2*; no GVBD), and control maturing oocytes at the first meiotic metaphase (lane *3*) with anti-starfish cdc25 (*upper panel*), anti-starfish cdc2 (*middle panel*) and anti-phosphorylated Tyr (at 34 kD; *lower panel*) antibodies. In lane *4*, the lysate from oocytes equivalent to lane *2* was treated with alkaline phosphatase (100 U/ml, for 60 min). In lane *5*, after 6-dimethylaminopurine (6-DMAP; final concentration, 5 mM in an oocyte) was coinjected into immature oocytes with the anti-cdc25 antibody, the recipients were treated with 1-MeAde for 30 min, but no GVBD occurred.

Production of Triggered Oocytes by the Use of Neutralizing Anti-cdc25 Antibody

To examine the regulation of the inactivator for cdc2 kinase, we noticed that our preparation of the anti-starfish cdc25 antibody, which was raised against the reported catalytic domain (Galaktionov and Beach, 1991), neutralized the activity of cdc25 phosphatase. As shown in Fig. 3 *A*, the anti-cdc25 antibody actually prevented the in vitro activation of the purified inactive form of cyclin B/p34^{cdc2} complex by the affinity-purified cdc25 phosphatase.

In accordance with the in vitro effect, when immature starfish oocytes were injected with the purified anti-cdc25 antibody, and then treated with 1-MeAde, GVBD was prevented in a manner dependent on the amount of injected antibody (Fig. 3 *B*). Complete inhibition was observed at 20 pg of the anti-cdc25 antibody, while control injection of the same amount of IgG had little effect on reinitiation of meiosis. The timing of GVBD was delayed by the injection of intermediate amount of the antibody. The inhibitory effect of the anti-cdc25 antibody was overcome by preincubation with cdc25 protein that was expressed in *E. coli* (data not shown). When the anti-cdc25 antibody prevented 1-MeAde–induced GVBD in vivo, no increase in histone H1 kinase activity was detected (Fig. 3 *C*). In these oocytes, the cdc2-L, which represents active form, was undetectable, and the phosphorylated form of the Tyr residue was still detectable at 34 kD (Fig. 3 *D middle and lower panels,* lane *2*). Therefore, the injected anti-cdc25 antibody indeed neutralized the activity of cdc25 phosphatase and thereby prevented the activation of cdc2 kinase after 1-MeAde addition. Thus, we could produce a triggered oocyte of an intermediate state, in which some of the pathways initiated by 1-MeAde might have already been triggered (see below), but cdc2 kinase was not yet activated.

In contrast to no apparent change in the electrophoretic mobility of cdc2 protein, a slight retardation occurred in cdc25 protein in triggered oocytes (Fig. 3 *D upper panel,* compare lane *2* with lanes *1* and *3*). The slight mobility shift of cdc25 protein in triggered oocytes was reversed by the treatment of oocyte lysates with alkaline phosphatase, which also caused the appearance of the cdc2-L and the disappearance of the phosphorylated Tyr residue (Fig. 3 *D* lane *4*). Further, the slight mobility shift of cdc25 protein was prevented by coinjection of 6-dimethylaminopurine, a potent kinase inhibitor, with the anti-cdc25 antibody (Fig. 3 *D* lane *5*). Thus, 1-MeAde addition appears to induce some modification, presumably phosphorylation, of the cdc25 protein even in the absence of the activation of cdc2 kinase, suggesting the presence of a kinase that phosphorylates cdc25 protein independently of the activation of cdc2 kinase. However, because of the low amount of cdc25 protein that can be recovered from the triggered oocytes, at present it was practically difficult to determine the phosphorylation sites or to measure the activity of cdc25 phosphatase in vitro.

Suppression of Inactivator for Cyclin B–dependent cdc2 Kinase in Triggered Oocytes

To examine whether the inactivator for cdc2 kinase is suppressed in triggered oocytes, subthreshold levels (see also Fig. 5 *A*) of the purified cdc2 kinase were injected into either triggered oocytes or intact immature oocytes, and then the fate of the injected cdc2 kinase was compared among these recipients. In intact immature oocytes (100 pl injection of starfish cdc2 kinase), when compared with the oocytes just after the injection, the recipient oocytes at 30 min later contained less than half a level of histone H1 kinase activity (Fig. 4 *A*, bars *1* and *2*). This indicates, in ac-

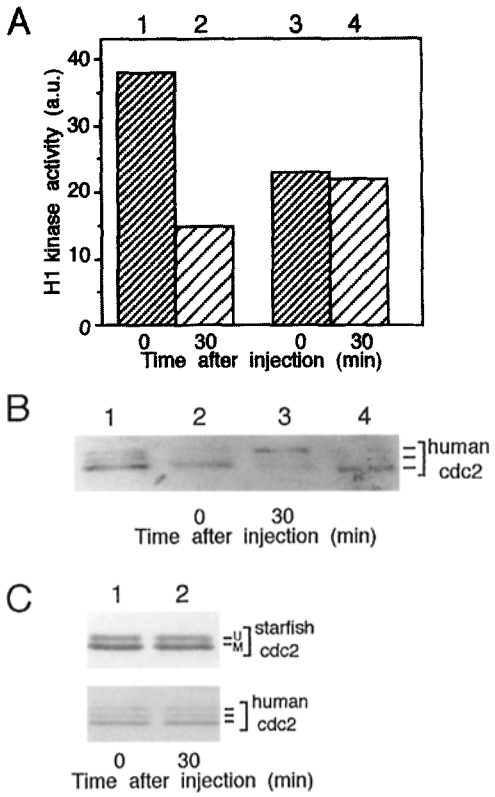

Figure 4. Subthreshold levels of cdc2 kinase are inactivated in recipient immature starfish oocytes but not in triggered oocytes. (*A*) Changes in the histone H1 kinase activity which was introduced by the injection of starfish cdc2 kinase. Immature starfish oocytes (bar *1*) or triggered oocytes (bar *3*) were injected with subthreshold levels of purified starfish cdc2 kinase (100 pl or 75 pl, respectively; see Fig. 5 *A*), and the levels of histone H1 kinase activity in recipients were measured just after the injection (0 min, bars *1* and *3*) and 30 min later (bars *2* and *4*). (*B*) Electrophoretic mobility shift-up of human cdc2 protein which was introduced into immature starfish oocytes. Subthreshold level of purified human cdc2 kinase was injected into immature starfish oocytes, and the changes of electrophoretic mobility of injected human cdc2 protein were detected by Western blots just after the injection (0 min, lane *2*) and 30 min later (lane *3*). Thereafter, the recipient oocytes were treated with 1-MeAde to induce GVBD, and the fate of human cdc2 protein in these oocytes was monitored (lane *4*). Lane *1* indicates purified human cdc2 preparation before the injection. (*C*) No modification occurs both in intrinsic starfish cdc2 protein and in exogenously introduced human cdc2 protein in recipient triggered oocytes. Subthreshold level of purified human cdc2 kinase was injected into triggered oocytes. Western blots of oocyte lysates with the anti-starfish cdc2 antibody (*upper panel*) or the anti-human cdc2 antibody (*lower panel*) just after the injection (0 min, lane *1*) or 30 min later (lane *2*).

cordance with Picard et al. (1991) who used cdc2 kinase that might have contained suc1 protein, that the exogenously introduced cdc2 kinase free of suc1 and cdc25 proteins was inactivated in recipient oocytes that failed to undergo GVBD. To confirm further the inactivation of the injected cdc2 kinase, subthreshold levels of the human cdc2 kinase, which could be distinguished from the endogenous starfish cdc2 protein, were injected into intact immature starfish oocytes (Fig. 4 *B*). The active form of human cdc2 protein represented by the fastest mobility (see Atherton-Fessler et al., 1994) was converted within 30 min to the inactive form represented by the slowest mobility (Fig. 4 *B*, lanes *2* and *3*). The inactivation of human cdc2 kinase was reversible, since the inactive form of human cdc2 protein was again converted to the active form when oocytes were treated with 1-MeAde 30 min after injection (Fig. 4 *B*, lane *4*). This reactivation indicates that the inactivation of cdc2 kinase was not due to proteolytic degradation, and clearly demonstrates that the human cdc2 kinase, which had been inactivated in immature starfish oocytes, was still responsive to the active starfish cdc25 phosphatase.

In contrast to intact immature oocytes, in triggered oocytes, the histone H1 kinase activity that was introduced by the injection of subthreshold levels (75 pl) of starfish cdc2 kinase suffered neither decrease nor increase by 30 min after injection (Fig. 4 *A*, bars *3* and *4*). When human cdc2 kinase was injected similarly, no change was observed in electrophoretic mobility of either intrinsic starfish or exogenous human cdc2 proteins in recipient triggered oocytes that did not undergo GVBD (Fig. 4 *C*). At 30 min after injection, the active form of human cdc2 protein was still detected at the same level as that seen just after the injection, while starfish cdc2 protein was maintained in the inactive form of cdc2-U. Also, no change in either cdc2 protein was observed in triggered oocytes when cdc2 kinase was injected together with butyrolactone I (Kitagawa et al., 1993), a potent inhibitor of cdc2 kinase action (Okumura, E., unpublished). Without 1-MeAde addition, however, microinjection of the anti-cdc25 antibody did not prevent the inactivation of cdc2 kinase that was injected into immature oocytes (data not shown), excluding the possibility that the anti-cdc25 antibody directly suppressed the inactivator for cdc2 kinase. These results indicate that in the absence of active cdc2 kinase, the inactivator for cdc2 kinase is suppressed in triggered oocytes, suggesting the presence of a suppressor for the inactivator of cdc2 kinase in triggered oocytes although the basis for this suppression is not yet known.

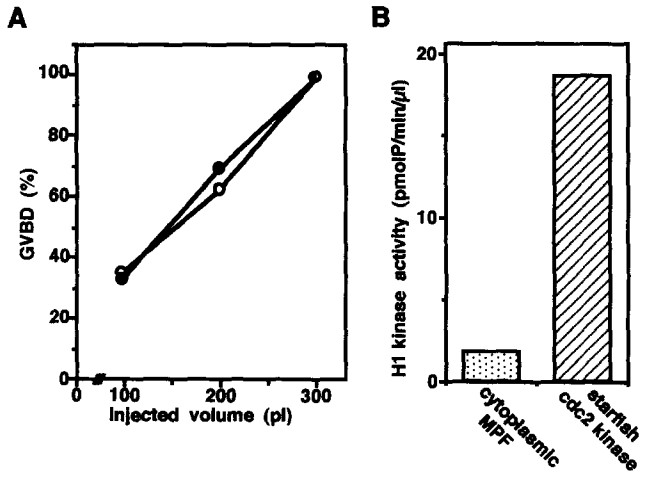

Figure 5. The level of histone H1 kinase activity necessary for GVBD induction in immature starfish oocytes is much higher in the purified cdc2 kinase preparation than in cytoplasmic MPF. (*A*) Dose dependency of GVBD induction on the injected amounts of cytoplasmic MPF (○) or the purified cdc2 kinase (●). The starfish cdc2 kinase preparation purified with $p13^{suc1}$-affinity column was concentrated so that 300 pl injection of it was just sufficient to induce GVBD at 100% in recipient starfish oocytes. Ten *Asterina* oocytes were injected for each point. (*B*) Levels of histone H1 kinase activity required for either cytoplasmic MPF or purified cdc2 kinase to induce 100% GVBD by injection into immature starfish oocytes. The histone H1 kinase activity contained in cytoplasmic MPF or the cdc2 kinase preparation shown in *A* was measured, respectively. In case of cytoplasmic MPF, the histone H1 kinase activity was estimated from that contained in starfish oocytes at the first meiotic metaphase (see Materials and Methods).

Meiosis-reinitiating Activity of Cyclin B–dependent cdc2 Kinase

Considering that the inactivator for cdc2 kinase is suppressed in triggered oocytes, it would be plausible that the levels of cdc2 kinase activity required for meiosis-reinitiation after injection might be less in triggered oocytes than in intact immature oocytes. To access this issue, we first checked whether the purified cdc2 kinase is precisely identical with the original cytoplasmic MPF in the aspect of the levels of H1 kinase activity that is required for GVBD induction in intact immature oocytes. When cytoplasm taken from 1-MeAde–treated starfish oocytes at the first meiotic metaphase was injected into immature starfish oocytes, ~300 pl of the cytoplasm, i.e. cytoplasmic MPF, which corresponds to ~1/13 of an oocyte volume, was a minimum requirement to induce GVBD in recipients at a rate of almost 100% (Fig. 5 *A*). Then, the starfish cdc2 kinase preparation purified with $p13^{suc1}$-affinity column was concentrated so that its injection of 300 pl was just enough to induce 100% GVBD in recipient starfish oocytes (Fig. 5 *A*). The percentage of oocytes that underwent GVBD decreased when smaller volumes of either purified cdc2 kinase or cytoplasmic MPF were injected. Despite similar activity in oocytes, however, cytoplasmic MPF and purified cdc2 kinase preparations contained extremely different levels of histone H1 kinase activity. In the minimum volume (300 pl) required to induce 100% GVBD in recipients, the purified starfish cdc2 kinase preparation had an ~10-fold higher level of histone H1 kinase activity than that contained in cytoplasmic MPF (Fig. 5 *B*). Similar levels of difference were still observed when cytoplasmic MPF was coinjected with the buffer for cdc2 kinase preparation (data not shown), or when histone H1 kinase activity of the purified cdc2 kinase was measured immediately after the injection into immature oocytes (Fig. 6 *A*, compare bars *3* with *1*), indicating that the discrepancy is not due to the difference between oocyte homogenates and purified preparations.

After injection of either cytoplasmic MPF or the starfish

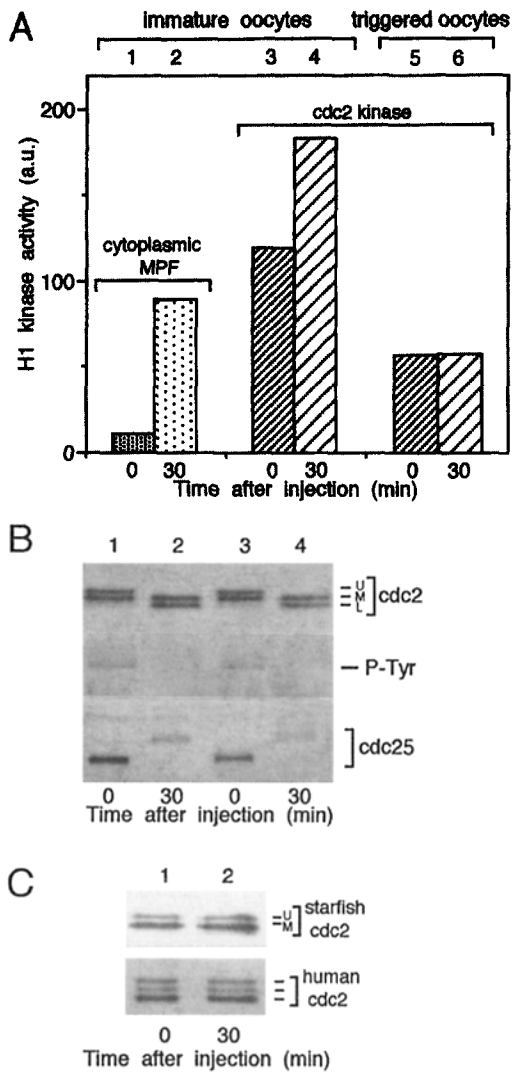

Figure 6. The level of cdc2 kinase activity required for GVBD induction is less in triggered oocytes than in intact immature oocytes in spite of the lack of amplification. (*A*) Changes in histone H1 kinase activity at GVBD after injection with either cytoplasmic MPF or cdc2 kinase into intact immature starfish oocytes or triggered oocytes. Either cytoplasmic MPF (bar *1*) or the starfish cdc2 kinase preparation indicated in Fig. 5 *A* (bar *3* for 300 pl; bar *5* for 150 pl) was injected into either intact immature starfish oocytes (bars *1* and *3*) or triggered oocytes (bar *5*). Levels of histone H1 kinase activity contained in these recipients were measured just after the injection (0 min; bars *1*, *3*, and *5*) or at the time of GVBD after injection (30 min, bars *2*, *4*, and *6*). (*B*) Intrinsic cdc25 phosphatase and cdc2 kinase are activated at GVBD after injection into immature starfish oocytes with either cytoplasmic MPF or purified human cdc2 kinase. When injection of either cytoplasmic MPF (lanes *1* and *2*) or human cdc2 kinase (lanes *3* and *4*) induced GVBD in intact immature starfish oocytes, mobility shifts in starfish cdc2 (*upper panel*) and cdc25 (*lower panel*) proteins, and the presence or absence of phosphorylated Tyr-residue at 34 kD (*middle panel*) were monitored in recipient starfish oocytes by Western blots. The upper band of cdc2, cdc2-U, which was present just after the injection (0 min, lanes *1* and *3*), disappeared and the lower band of cdc2, cdc2-L, appeared reciprocally at the time of GVBD after injection (30 min, lanes *2* and *4*). (*C*) No modification occurs both in intrinsic starfish cdc2 protein and in exogenously introduced human cdc2 protein in recipient triggered oocytes at GVBD. The purified human cdc2 kinase was injected into triggered oocytes to induce GVBD as indicated in *A*. Western blots of oocyte lysates with the anti-starfish cdc2 antibody (*upper panel*) or the anti-human cdc2 antibody (*lower panel*) just after the injection (0 min, lane *1*) or 30 min later (lane *2*).

cdc2 kinase preparation, so-called amplification, i.e., the activation of the cyclin B/p34^{cdc2} complex and cdc25 phosphatase which were already present as an inactive form in recipient immature oocytes, occurred: at the time of GVBD after the injection, histone H1 kinase activity increased by similar amount in both cases of recipients (Fig. 6 *A*, bars *2* and *4*). Regardless of whether cytoplasmic MPF or purified human cdc2 kinase induced GVBD, the starfish cdc2-U and the phosphorylated form of Tyr residue at 34 kD disappeared, and the starfish cdc2-L, which represents the active form, appeared in both recipient oocytes after GVBD (Fig. 6 *B*, *upper and middle panels*, lanes *1–4*). In accordance with these, the electrophoretic mobility of cdc25 protein was retarded to the same level in both recipients (Fig. 6 *B*, *lower panel*, lanes *1–4*), indicating the activation of intrinsic cdc25 phosphatase.

By contrast, in triggered oocytes, microinjection of 150 pl of the purified starfish cdc2 kinase, which corresponds to only one-half the level of histone H1 kinase activity required for intact immature oocytes, was sufficient to induce 100% GVBD, even though no appreciable increase of histone H1 kinase activity was observed in recipient oocytes (Fig. 6 *A*, bars *5* and *6*). The lack of amplification in recipient triggered oocytes was confirmed by the presence of starfish cdc2-U and the absence of starfish cdc2-L when GVBD was induced by the injection of human cdc2 kinase (Fig. 6 *C upper panel*). In this case, no change was also observed in human cdc2 protein (Fig. 6 *C lower panel*). In contrast that GVBD was completed within 30 min after 1-MeAde addition in control immature oocytes, the timing of GVBD was delayed in triggered oocytes with the decrease in injected amounts of cdc2 kinase (data not shown). The observed correlation may reflect the actual response of oocytes to the injected cdc2 kinase, since neither amplification nor loss of cdc2 kinase activity occurs in triggered oocytes.

These observations suggest that the discrepancy between cytoplasmic MPF and purified cdc2 kinase in the levels of histone H1 kinase activity required for GVBD is at least partially explained by the ability of cytoplasmic MPF to suppress the inactivator of cdc2 kinase. Since previous studies (Kishimoto et al., 1981; Picard and Doree, 1984) suggest the involvement of germinal vesicle components in the MPF activity that is detectable by injection into immature starfish oocytes, we examined whether the suppression of the inactivator for cdc2 kinase depends on the presence of germinal vesicle. We applied the procedure for the production of triggered oocytes to immature oocytes that had been enucleated previously. In these triggered enucleated oocytes as well, sub-threshold levels of the injected human cdc2 kinase were not inactivated (Fig. 7), while the inactivation of cdc2 kinase was observed in control enucleated immature oocytes (data not shown). These facts imply that in triggered oocytes, the suppression activity for the inactivator of cdc2 kinase does not depend on germinal vesicle components.

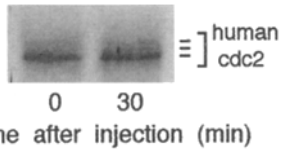

Figure 7. The inactivator for cdc2 kinase is suppressed independently of germinal vesicle in triggered oocytes. Immature starfish oocytes were enucleated, injected with the neutralizing anti-cdc25 antibody, and then treated with 1-MeAde. These oocytes were injected with subthreshold level of human cdc2 kinase. Human cdc2 protein in recipients was monitored by Western blots just after its injection (*0 min*) or 30 min later. Note that no change occurs in the lower band of human cdc2 protein.

Discussion

Components Controlling the Oocyte G2 Checkpoint

By using the purified cdc2 kinase free of suc1 and cdc25 proteins, and the neutralizing anti-cdc25 antibody, the present study demonstrates clearly that the activity of cdc2 kinase at the oocyte G2 checkpoint is regulated by a network that includes at least the following components: an inactivator for cdc2 kinase, a suppressor of this inactivator, cdc25 phosphatase, a putative kinase which phosphorylates cdc25 protein independently of cdc2 kinase, and a phosphatase which dephosphorylates cdc25 protein (see Fig. 8). Candidates for these activities are as follows.

The inactivator for cdc2 kinase might include at least wee1-like kinase and/or the Thr14/Tyr15 kinase. In the suppression of wee1-like kinase, Devault et al. (1992) suggest the involvement of cyclin A-dependent kinase. But, this possibility is excluded in the present suppression, because cyclin A is undetectable until first meiotic metaphase in starfish oocytes (Okano, T., and T. Kishimoto, manuscript in preparation). Nonetheless, dual type of phosphorylation-dependent inactivation is suggested in the downregulation of wee1 kinase at M-phase (Coleman et al., 1993; Tang et al., 1993; for review see Coleman and Dunphy, 1994). Fission yeast nim1/cdr1 kinase phosphorylates its COOH-terminal domain, while M-phase extracts of *Xenopus* eggs contain at least two responsible kinases for NH_2-terminal domain (Mueller et al., 1995); cdc2 kinase showing direct effect and a distinct kinase related to a putative MPM-2 epitope kinase (ME kinase) (Kuang and Ashorn, 1993; Westendorf et al., 1994). However, human wee1 kinase is phosphorylated but not inactivated by cdc2 kinase (Watanabe et al., 1995), and in the present study the suppression of the inactivator for cdc2 kinase was accomplished in the absence of cdc2 kinase and cdc25 phosphatase activities (Figs. 3 and 4). On the other hand, at present the suppression mechanism of the Thr14/Tyr15 kinase is unclear except that its activity is undetectable at M-phase (Atherton-Fessler et al., 1994; Kornbluth et al., 1994). Taken together, the putative suppressor for the inactivator of cdc2 kinase would be an unknown inhibitory kinase, possibly related to the ME kinase.

The slight mobility shift-up of cdc25 protein, which was observed in triggered oocytes, is first revealed at the initiation of M-phase in vivo, but in the absence of the activation of cdc2 kinase. As a candidate for the responsible kinase, the possibility of cyclin A–dependent cdc2 kinase is again excluded due to its absence (see above). In interphase extracts of *Xenopus* eggs, phosphatase inhibitors cause phosphorylation and activation of cdc25 phosphatase in the absence of cdc2 kinase activity (Izumi et al., 1992; Kumagai and Dunphy, 1992; Clarke et al., 1993; Izumi and Maller, 1995). As a candidate for a cdc25 protein-phosphorylating kinase that is distinct from cdc2 kinase, the ME kinase has been again suggested (Kuang and Ashorn, 1993; Kuang et al., 1994; Izumi and Maller, 1995). Taken together, an unknown kinase, presumably related to the ME kinase, would be involved in the initial phosphorylation of cdc25 protein at the release from G2-phase arrest, while a type-2A phosphatase might counteract this phosphorylation (see Izumi et al., 1992; Clarke et al., 1993; Izumi and Maller, 1995).

In Vivo Coordination for the Initial Activation of cdc2 Kinase at the Release from G2-Phase Arrest of Oocytes

How are the regulatory balances coordinately tipped to-

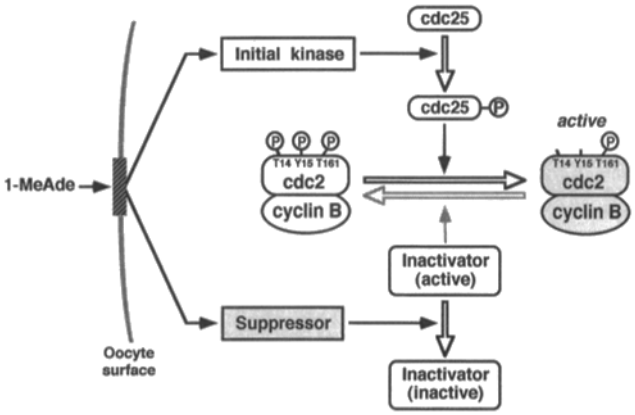

Figure 8. A model for the initial activation of cdc2 kinase in vivo at the release from the oocyte G2-phase checkpoint. The G2-phase arrest of immature oocytes is ensured by an active form of an inactivator of cdc2 kinase. 1-MeAde treatment of oocytes causes the release from the G2-phase arrest by two pathways that are independent of the cdc2 kinase activity: In one pathway, the activity of the inactivator of cdc2 kinase is downregulated by its suppressor before the cdc2 kinase activation, whereas in the other pathway, cdc25 undergoes an initial cdc2 kinase-independent phosphorylation by an initial kinase that may activate cdc25 phosphatase activity. Activation of these two pathways in response to the maturation-inducing stimulus, 1-MeAde, results in the initial increase in cdc2 kinase activity by simultaneously removing an inhibitor of the kinase and stimulating the activation of previously inactive cyclin B/$p34^{cdc2}$ complexes. Further increase in cdc2 kinase activity may be through the positive amplification loop described by others (see text for details). The inactivator of cdc2 kinase seems to include the Thr14/Tyr15 kinase for $p34^{cdc2}$ and/or wee1/mik1 kinase. Although the components of activities involved in the suppressor for the inactivator of cdc2 kinase and the initial phosphorylation of cdc25 protein are not yet known, it is likely that they include unknown protein kinases. Accordingly, the upregulation of these protein kinases and/or the downregulation of phosphatases that counteract these kinases, presumably type-2A phosphatases, contribute to the suppressor and the initial kinase activity. We propose that, in addition to cdc2 kinase activity, the activity of the suppressor for the inactivator of cdc2 kinase is also a component of MPF detected by cytoplasmic transfer experiments. See text for details.

wards the initial activation of cdc2 kinase in vivo? Because of the production of triggered oocytes, the present study clearly brings the answer to this question at least on the release from the G2 checkpoint of oocytes. As summarized in Fig. 8, before the initial activation of cdc2 kinase, its inactivator is actually downregulated by the suppressor, and coincidentally cdc25 phosphatase may be upregulated by its initial kinase. It may be possible that both the suppressor and the initial kinase are the same ME kinase that is under the control of 1-MeAde stimulus.

Alternatively, the present study indicates that a phosphatase, presumably type-2A, counteracts the unknown kinase in the initial phosphorylation of cdc25 protein (see above in relation to Fig. 2 *B*). The type-2A phosphatase is also involved in the upregulation of wee1 kinase (Muller et al., 1995; McGowan and Russell, 1995). These imply that the downregulation of type-2A phosphatase would cause both the upregulation of cdc25 phosphatase and the downregulation of wee1 kinase, serving as both the initial kinase of cdc25 protein and the suppressor for the inactivator of cdc2 kinase. Thus, either the upregulation of a presumed ME kinase or the downregulation of a presumed type-2A phosphatase, or both might initiate the cdc2 kinase activation at the release from the oocyte G2 checkpoint.

The present result implys that both the inactivator of cdc2 kinase and the phosphatase of cdc25 protein are still able to exhibit their activities during the hormone-dependent period even after the activation of cdc2 kinase has been already initiated (Fig. 2 *B*). The hormone-dependent period might correspond to a duration before both regulators lose their activity. These notions do not suggest that both the suppressor for the inactivator of cdc2 kinase and the initial kinase of cdc25 protein have completed their functions before the initial activation of cdc2 kinase, but rather that both accomplish their functions in parallel with the activation of cdc2 kinase. Taken together, there might be two distinct processes for the cdc2 kinase activation: (*1*) a cdc2 kinase activity–independent one including both the suppressor for the inactivator of cdc2 kinase and the initial kinase of cdc25 protein, and (*2*) a cdc2 kinase activity–dependent one including the feedback loops positively with cdc25 phosphatase (Hoffmann et al., 1993; Izumi and Maller, 1993) and negatively with the inactivator of cdc2 kinase (see Mueller et al., 1995). At the most initial triggering of M-phase, the cdc2 kinase activity–independent process might trip the switch that leads to the initial activation of cdc2 kinase, and thereafter, both processes might proceed in parallel possibly during the hormone-dependent period. After such an initial step, feedback that depends on the activity of cdc2 kinase might further increase the cdc2 kinase activity.

Responsible Components for Cytoplasmic MPF

MPF was originally identified as a cytoplasmic activity that reinitiates meiosis upon injection into immature oocytes. Cdc2 kinase is without doubt an essential component of cytoplasmic MPF (Lohka et al., 1988; Gautier et al., 1988, 1990; Labbe et al., 1989; present study). If cdc2 kinase were the only responsible component for the original cytoplasmic MPF, the level of histone H1 kinase activity that is required for the induction of meiosis reinitiation would be similar between cytoplasmic MPF and cdc2 kinase. In this issue, however, our present data (Fig. 5 *B*) clearly demonstrates the discrepancy, indicating that other component(s) than cdc2 kinase is responsible for MPF activity (see also Picard et al., 1991).

What is a responsible component(s) for cytoplasmic MPF? Comparison of control immature oocytes with triggered oocytes (Fig. 6 *A*, bars *3* and *5*) indicates that the level of cdc2 kinase required for meiosis reinitiation is much less when the inactivator of cdc2 kinase is suppressed. In the absence of the suppression, a higher level of cdc2 kinase might be necessary to overcome the inactivator and to induce meiosis reinitiation. If so, the suppressor for the inactivator of cdc2 kinase would be a possible candidate for the responsible component of cytoplasmic MPF.

In starfish oocytes, previous studies indicate that germinal vesicle contents are required for the cytoplasmic MPF activity that is detectable upon injection into immature oocytes (Kishimoto et al., 1981; Picard and Doree, 1984), in spite that after 1-MeAde addition enucleated oocytes exhibit similar levels of histone H1 kinase activity as nucleated oocytes (Picard et al., 1988). In this context, we could produce triggered oocytes even from previously enucleated immature oocytes (Fig. 7), implying that the inactivator of cdc2 kinase is suppressed independently of the presence of germinal vesicle. We conclude that the present suppressor for the inactivator of cdc2 kinase is distinct from the germinal vesicle components required for MPF activity and is a previously unrecognized component of MPF.

Cdc25 phosphatase alone exhibits MPF activity (Gautier et al., 1991; Kumagai and Dunphy, 1991) and associates with cdc2 kinase (Jessus and Beach, 1992; present study), suggesting that cdc25 phosphatase may constitute cytoplasmic MPF. However, the cdc2 kinase level that was required for meiosis reinitiation was much less in triggered oocytes where cdc25 phosphatase was prevented than in control immature oocytes where it was able to be activated (Fig. 6 *A*, bars *3* and *5*). Thus, cdc25 phosphatase would not be a necessary but rather a supplementary component for cytoplasmic MPF.

In summary, we propose a hierarchy for the components of cytoplasmic MPF: the minimal essential component is cdc2 kinase; the secondary responsible component is the suppressor for the inactivator of cdc2 kinase; and the supplementary component is cdc25 phosphatase.

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