

A Complex Containing p34^{cdc2} and Cyclin B Phosphorylates the Nuclear Lamin and Disassembles Nuclei of Clam Oocytes in Vitro

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Abstract. Cell-free extracts prepared from activated clam oocytes contain factors which induce phosphorylation of the single 67-kD lamin (L67), disassemble clam oocyte nuclei, and cause chromosome condensation in vitro (Dessev, G., R. Palazzo, L. Rebhun, and R. Goldman. 1989. *Dev. Biol.* 131:469–504). To identify these factors, we have fractionated the oocyte extracts. The nuclear lamina disassembly (NLD) activity, together with a protein kinase activity specific for L67, appear as a single peak throughout a number of purification steps. This peak also contains p34^{cdc2}, cyclin B, and histone H1-kinase activity, which are com-

ponents of the M-phase promoting factor (MPF). The NLD/L67-kinase activity is depleted by exposure of this purified material to Sepharose conjugated to p13^{suc1}, and is restored upon addition of a p34^{cdc2}/p62 complex from HeLa cells. The latter complex phosphorylates L67 and induces NLD in the absence of other clam oocyte proteins. Our results suggest that a single protein kinase activity (p34^{cdc2}-H1 kinase, identical with MPF) phosphorylates the lamin and is involved in the meiotic breakdown of the nuclear envelope in clam oocytes.

DURING mitosis, the nuclear envelopes of most eukaryotic cell types are disassembled. One biochemical reaction that is believed to be important for nuclear envelope breakdown (NEBD)¹ is the phosphorylation of the nuclear lamins, which are major protein components of the nuclear lamina (NL) (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985; Dessev and Goldman, 1988; Dessev et al., 1989). Therefore, NEBD is likely to involve a cell cycle-regulated lamin-specific protein kinase.

NEBD, as well as other M-phase specific events, is under control of an activity termed M-phase promoting factor (MPF), which is abundant in mitotic but not in interphase cells (Masui and Markert, 1971; Smith and Eckert, 1971; Wasserman and Smith, 1978; Sunkara et al., 1979; Kishimoto et al., 1982; for reviews see Murray and Kirschner, 1989a; Lohka, 1989; Nurse, 1990). Microinjection of MPF into interphase cells causes chromosome condensation, NEBD, and assembly of the mitotic spindle (Masui and Markert, 1971; Smith and Eckert, 1971; Wu and Gerhart, 1980; Miake-Lye et al., 1983). The dependence of these M-phase events on MPF has been established also in cell-free systems

(Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985). It has been postulated that MPF initiates a pathway leading to activation of a lamin-specific kinase, which causes NL disassembly (NLD) by phosphorylating the nuclear lamins (Miake-Lye and Kirschner, 1985; Newport and Spann, 1987; Murray and Kirschner, 1989b).

Recently, MPF has been purified from oocytes of *Xenopus* and starfish (Lohka et al., 1988; Arion et al., 1988; Labbe et al., 1989). It has been found to contain a 34-kD protein kinase homologous to the *cdc2* gene product (p34^{cdc2}), which is involved in cell cycle regulation in fission yeast (Dunphy et al., 1988; Gautier et al., 1988; Nurse, 1985; Hayles and Nurse, 1986). MPF appears to be identical with the previously described growth-associated histone H1 kinase (Bradbury et al., 1974; Langan et al., 1989).

The second major component of MPF is cyclin. Cyclins are synthesized and accumulate throughout the cell cycle and undergo abrupt degradation at the end of mitosis (Evans et al., 1983; Swenson et al., 1986; Murray et al., 1989). They are required to activate the protein kinase function of p34^{cdc2} during mitosis presumably by forming complexes with it (Draetta et al., 1989; Westendorf et al., 1989; Labbe et al., 1989). Periodic synthesis and destruction of cyclins is sufficient to drive some embryonic cell cycle events (Murray and Kirschner, 1989b; Murray et al., 1989).

In the past ten years, little progress has been made towards

1. *Abbreviations used in this paper:* L67, the single 67-kD clam oocyte lamin; MPF, M-phase promoting factor; NL, nuclear lamina; NEBD, nuclear envelope breakdown; NLD, nuclear lamina disassembly.

identification of the protein kinase(s) involved in lamin phosphorylation *in vivo*. A number of protein kinases have been shown to phosphorylate lamins *in vitro* (Erikson and Maller, 1988; Dessev et al., 1988), but their nature has not been investigated. Quite recently, three studies relevant to this problem have been published. Heald and McKeon (1990) have shown that mutations at two highly conserved sites in human lamin A (Ser-22 and Ser-392) expressed in CHO cells block the disassembly of the NL during mitosis. Their findings are in agreement with the direct sequencing results of Ward and Kirschner (1990), who have studied the phosphorylation of bacterially expressed human lamin C in mitotic extracts from frog eggs. The enzyme responsible for the lamin phosphorylation in these experiments has not been identified, although the involvement of multiple kinases including the ribosomal S6 kinase II has been suggested on the basis of phosphopeptide analysis. Finally, Peter et al. (1990) have demonstrated that p34^{cdc2} protein kinase purified from starfish oocytes catalyses the incorporation of [³²P]phosphate into lamins B₁ and B₂ from chicken embryonic nuclei *in vitro* at sites similar to those modified during M-phase *in vivo*, and induces solubilization of these lamins. These results, however, have been obtained using systems consisting of heterologous components. Therefore, they have identified enzymes that can phosphorylate the nuclear lamins, but have furnished little direct information concerning the nature of the enzyme(s) that do modify these proteins *in vivo*.

To understand the mechanisms by which MPF controls NEBD, we have studied the activity involved in NLD and lamin phosphorylation in oocytes of the clam, *Spisula solidissima* (Dessev et al., 1989). These oocytes are arrested at the end of the first meiotic prophase (Allen, 1953). Within 10–12 min after fertilization or parthenogenic stimulation, the oocytes develop an MPF activity (Kishimoto et al., 1984) and undergo NEBD (Allen, 1953). We have shown that this is accompanied by an increase in the phosphate content of the single 67-kD oocyte lamin (L67) by 2–3 mol/mol protein, and its release from the NL polymer as a mixture of monomers and dimers (Dessev and Goldman, 1988; Dessev et al., 1990). We have also developed a homologous cell-free system containing an extract from activated oocytes, in which L67 phosphorylation and disassembly of purified oocyte nuclei occur in a manner similar to that *in vivo* (Dessev et al., 1989; Dessev et al., 1990).

Clam oocytes and embryos contain a homologue of p34^{cdc2}. In oocytes, it is associated with cyclin B in the form of an inactive complex (Shibuya, E., and J. Ruderman, manuscript in preparation) whose activation appears to involve dephosphorylation of p34^{cdc2} (Dessev, G., unpublished data). In embryos, p34^{cdc2} is associated with both cyclin A and B in complexes that display a cell cycle-dependent histone H1 kinase activity and function as MPF during oocyte maturation and embryogenesis (Draetta et al., 1989; Westendorf et al., 1989).

In this study, we have purified a protein complex, which contains p34^{cdc2}, cyclin B, and H1 kinase activity. This complex, which is indistinguishable from MPF, phosphorylates L67 *in vitro* at a set of sites similar to those modified *in vivo* and directly causes isolated oocyte nuclei to undergo NLD and chromosome condensation.

Materials and Methods

Animals, Oocytes, and Cytosolic Extracts

Surf clams were obtained from the Department of Marine Resources at Marine Biological Laboratory, Woods Hole, MA. The oocytes were handled as previously described (Dessev and Goldman, 1988). Cell-free extracts active in NLD, and detergent-purified oocyte nuclei (NP-40 nuclei) were prepared as described in Dessev et al. (1989).

Antibodies

The rabbit polyclonal serum against L67 (Pab227) was described previously (Dessev and Goldman, 1988; Dessev et al., 1989). The polyclonal antibody against yeast p34^{cdc2} (G8) was raised in rabbits using protein expressed and purified from *Escherichia coli* (Booher et al., 1989). The rabbit polyclonal serum used for identification of the clam cyclin B has been described earlier (Westendorf et al., 1989).

Labeling of L67 *In Vivo*

To generate an intracellular pool of radioactive ATP, the oocytes were incubated in the presence of inorganic [³²P]phosphate for 3 h, then activated in 1.4 M glycerol containing 15 mM phosphate buffer, pH 8.0, and lysed as described in Dessev and Goldman (1988). The soluble L67 was recovered by immunoprecipitation.

Assay for NLD *In Vitro* and L67 Kinase Activity

NP-40 nuclei (1–2 × 10⁵/ml) were incubated at room temperature in the presence of cell-free extracts in buffer D (50 mM KCl, 40 mM beta-glycerophosphate, 10 mM EGTA, 5 mM MgCl₂, 2 mM DTT, 20 mM Pipes, pH 7.4) containing 0.2% BSA, 0.5–1 mM ATP, and an ATP-regenerating system (20 mM phosphocreatine and 50 μg/ml phosphocreatine kinase). 20-μl aliquots were placed in glass chambers for observation under a phase-contrast microscope coupled to a video camera and monitor. The NLD activity was measured as a function of the rate of nuclear shrinking, which reflects the rate of L67 solubilization (Dessev et al., 1989), or by measuring the time necessary for disassembly of 90% of the nuclei. The amount of solubilized L67 was determined by immunoblotting with Pab227 directly in the pellet and supernatant after centrifugation of the samples in an Eppendorf microfuge (Brinkman Instruments Co., Westbury, NY) at 14,000 rpm for 5 min.

To measure L67 phosphorylation, aliquots of the samples were incubated with NP-40 nuclei as described above, in the presence of 10–100 μCi/ml of [gamma-³²P]ATP. The samples were solubilized in hot SDS either directly or after separation into 14,000-rpm pellets and supernatants. L67 was recovered by immunoprecipitation. The immunoprecipitates were fractionated by SDS-PAGE, the gels were autoradiographed, and the bands containing L67 were excised and counted by Cerenkov radiation.

In some experiments, instead of nuclei, L67 immunoadsorbed on protein A-Sepharose-Pab 227 was used as a substrate for L67 kinase. To prepare this substrate, oocyte nuclei were dissolved in SDS sample buffer by boiling for 3 min, diluted with immunoprecipitation buffer, and immunoadsorbed on protein A-Sepharose beads presaturated with Pab 227. The catalytic subunit of the cAMP-dependent protein kinase was obtained from Sigma Chemical Co. (St. Louis, MO).

Assay for Histone H1 Kinase Activity

3 μl of the samples were incubated with 5 μg of histone H1 (Boehringer Mannheim Diagnostics Inc., Houston, TX) and 0.5 mM [gamma-³²P]ATP in a total volume of 20 μl buffer D/0.2% BSA for 20 min at room temperature. The reaction was stopped by addition of 5 μl SDS sample buffer (5×) and the samples were analyzed by SDS-PAGE and autoradiography. H1-containing bands were excised and counted by Cerenkov radiation. H1 kinase associated with p13-Sepharose was determined by incubation of the beads under the same conditions.

Immunoprecipitation and Immunoblotting

Samples were diluted to 1–2 ml with immunoprecipitation buffer (0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.2% BSA, and 20 mM Pipes-NaOH, pH 7.4), and rocked for 2–24 h at 4°C with a 20–50 μl suspen-

sion of protein A-Sepharose beads (Sigma Chemical Co.) presaturated with Pab 227. The beads were collected and washed four times with immunoprecipitation buffer. The adsorbed material was eluted with SDS sample buffer and analyzed by SDS-PAGE and autoradiography (Laemmli, 1970). The conditions for immunoblotting have been described previously (Dessev and Goldman, 1988; Dessev et al., 1989). The polyclonal antibodies against cyclin B and p34^{cdc2} were used at a dilution of 1:500, followed by 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology, Birmingham, AL).

Fractionation in Sucrose Density Gradients

5-ml 5–20% sucrose gradients were prepared in buffer D containing 1 M KCl, CHAPS, and 0.2% BSA. KCl and CHAPS were added to the samples to 1 M and 1%, respectively, and aliquots of 300 μ l were applied on the gradients. Centrifugation was for 16 h at 40,000 rpm (4°C) in a rotor (model SW 55Ti; Beckman Instruments, Inc., Palo Alto, CA). Catalase ($M_r = 232,000$) and BSA ($M_r = 67,000$) were run in a separate tube as molecular weight markers. Before analysis, 100- μ l aliquots of each gradient fraction were passed through 1-ml spin dry Sephadex G25 columns prepared in Eppendorf blue tips (Brinkman Instruments Co.) and equilibrated with buffer D/0.2% BSA.

Treatment with p13-Sepharose

Sepharose conjugated to p13^{suc1} was prepared as described in Arion et al. (1988). 50–100- μ l samples were shaken at 0°C for 30 min with equal volume of a 1:1 suspension of p13-Sepharose in buffer D/0.2% BSA. The beads were recovered by centrifugation, washed four times with buffer D, and used for further analyses. The material adsorbed on the beads was eluted with SDS sample buffer by boiling for 3 min. The p13-Sepharose-treated supernatant was analyzed for the presence of p34^{cdc2}, cyclin B, H1 kinase, L67 phosphorylation and solubilization, and NEBD activity. BrCN-activated Sepharose (Sigma Chemical Co.) was coupled to BSA according to the instructions of the manufacturer and used as a control to p13-Sepharose.

Phosphopeptide Mapping

L67 was phosphorylated *in vivo* as described above. For phosphorylation of L67 *in vitro*, standard conditions for cell-free NLD were used except that the reaction mixture contained 0.1–0.5 mM [γ -³²P]ATP and no ATP-regenerating system. In both cases L67 was recovered by immunoprecipitation and purified by SDS-PAGE. The gels were not stained; instead, they were soaked in 50% methanol (2 changes, 15-min each at room temperature) and dried between two sheets of cellophane. L67 fractions were localized by autoradiography and cut from the gels. The sections were extracted with 50 and 100% methanol for 2 h each (these extractions turned out to be essential for the good quality of the peptide maps), and dried. L67 was digested with trypsin (Sigma Chemical Co.; TPCK-treated, 100 μ g/ml in 50 mM (NH₄) HCO₃, pH 7.9, containing 2 mM DTT) for 24–36 h at 30°C. The hydrolysate was freeze-dried, and fractionated on thin cellulose layers (Kodak) by electrophoresis in acetic acid/formic acid/H₂O (45:20:435) for 60 min at 900 kV and 8°C, followed by ascending chromatography in *n*-butanol/acetic acid/pyridine/H₂O (60:12:40:48). The phosphopeptides were detected by autoradiography.

Isolation of p34^{cdc2}/p62 Complex from HeLa Cells

Human cdc2 protein kinase was purified from HeLa cells as described (Brihueza et al., 1989), except that after lysis by Dounce homogenization the lysate was adjusted to 0.5 M NaCl and further homogenized to salt extract the nuclei before centrifugation. This resulted in a 5–10-fold increase in the recovery of kinase activity (Bischoff, J., unpublished data).

Results

The Specificity of L67 Kinase *In Vitro* Is Similar to That *In Vivo*

Since the present experiments involve phosphorylation of L67 in cell-free extracts, it has been essential to compare the specificity of the protein kinase which modifies L67 *in vitro* (Dessev et al., 1989) and *in vivo* (Dessev and Goldman,

1988). To establish the *in vivo* pattern of L67 phosphorylation, oocytes were pre-labeled with [³²P]orthophosphate and activated. After NEBD was completed, L67 was recovered from the detergent-soluble fraction by immunoprecipitation and subjected to peptide analysis. *In vivo*-labeled L67 showed the presence of two major phosphopeptides, 1 and 2, and a small number of minor spots with varying intensities (Fig. 1 A). The same major phosphopeptides were seen in L67 labeled *in vitro* using crude oocyte extracts (Fig. 1, B and D). Interestingly, L67 solubilization seemed to be accompanied by an increase in the intensity of spot 2 and by disappearance of several minor spots seen in the maps of L67 remaining insoluble (Fig. 1 D, compare with B). These results suggest that the modification of the site corresponding to spot 2 is essential for NLD, and also that a protein phosphatase activity is present in these extracts (see Discussion).

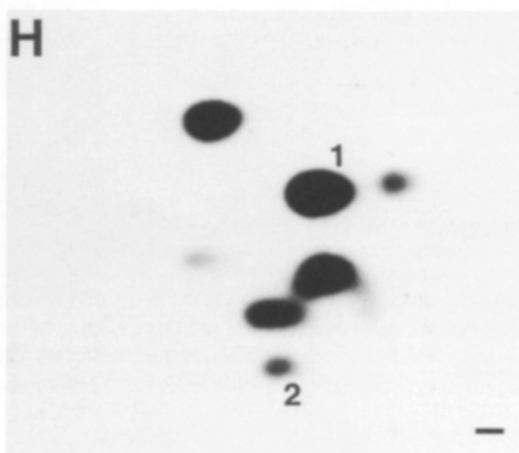
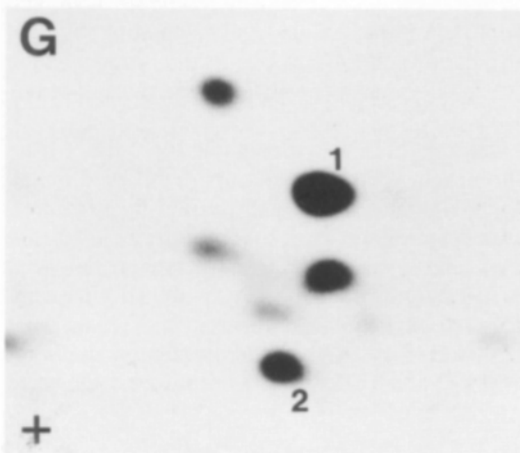
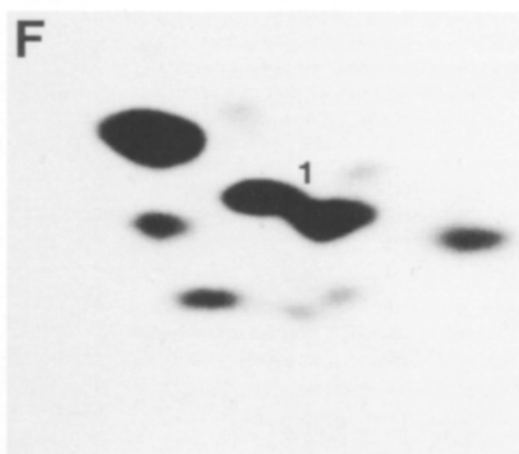
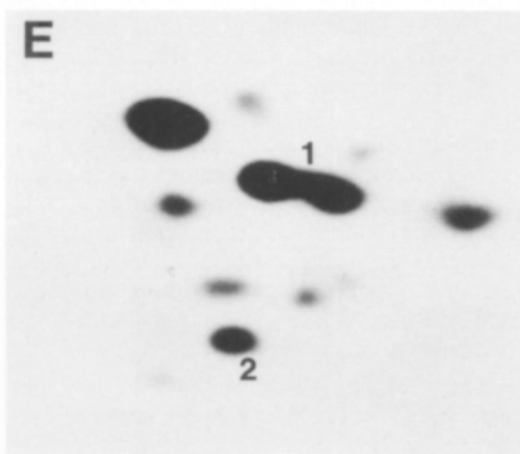
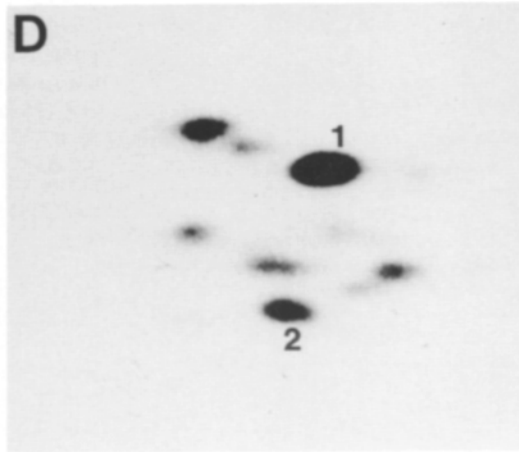
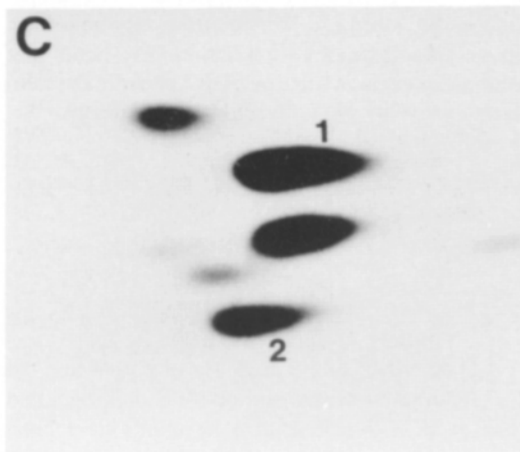
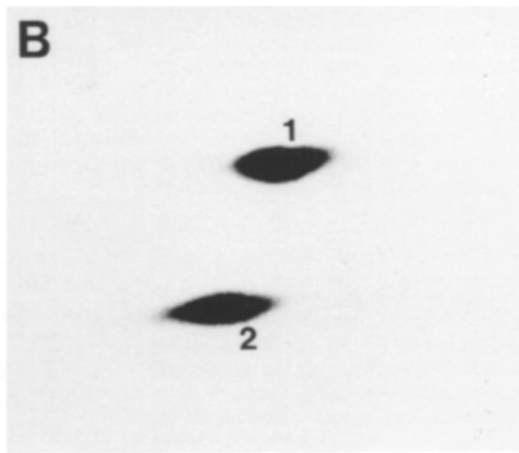
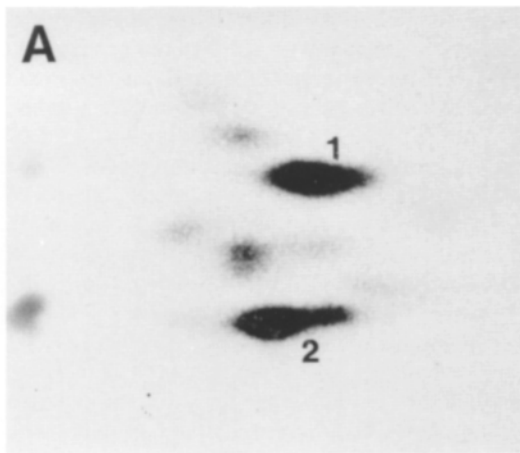
On the basis of the similarity between the *in vivo* and *in vitro* phosphopeptide maps of L67 it can be concluded that the same protein kinase is involved in L67 phosphorylation and NLD both *in vivo* and under cell-free conditions.

NLD/L67-Kinase Activity Copurifies with H1-Kinase and P34^{cdc2}/Cyclin B Complex

Crude extracts active in NLD and L67 phosphorylation (Dessev et al., 1989) were fractionated and assayed for (a) L67 phosphorylating activity using NP-40 nuclei or L67 immunoadsorbed on protein A-Sepharose beads as substrates; (b) *in vitro* NLD activity; (c) the presence of p34^{cdc2} and cyclin B; and (d) histone H1-kinase activity. It should be noted that the nuclei used in the present experiments have been depleted of the lipid components of the nuclear envelope (Dessev et al., 1989). Disassembly of these nuclei, therefore, refers to changes occurring with the NL, nuclear pore complexes, and chromosomes, but not with the nuclear membranes.

More than 90% of both L67 kinase and H1 kinase activities were recovered in the ammonium sulfate precipitate at 33% saturation. These activities were stabilized by incubation for 2–4 h at room temperature in buffer D containing 1 mM ATP- γ -S, an analogue of ATP which introduces phosphoryl groups resistant to phosphatases (Eckstein, 1975), or 1 mM ATP plus ATP-regenerating system. For this reason, all fractionation procedures were carried out in the presence of 0.5–1 mM ATP. The activity adhered to glass and plastic surfaces, which led to substantial losses. This problem was alleviated by including 0.2% BSA in the buffers used in the assays, and in the sucrose density gradients. Although the presence of BSA prevented us from calculating the final degree of purification achieved, it significantly improved the yield of NLD activity that was critical to the success of our experiments.

The 33% ammonium sulfate precipitate was further fractionated by ion-exchange chromatography, followed by gel filtration, and sedimentation in sucrose density gradients. H1 kinase was not retained on DEAE cellulose at 50 mM KCl (Fig. 2 A), and it was eluted from phosphocellulose at 0.45 M KCl (Fig. 2 B). From Sephacryl S-300 H1 kinase appeared as a broad peak with an apparent molecular mass higher than 200 kD (Fig. 2 C). In sucrose density gradients containing 1 M KCl and 0.5% CHAPS, H1 kinase sedimented



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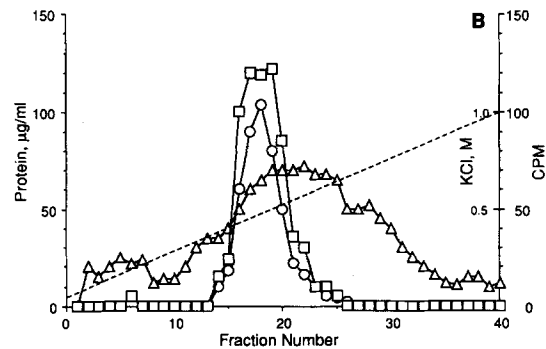
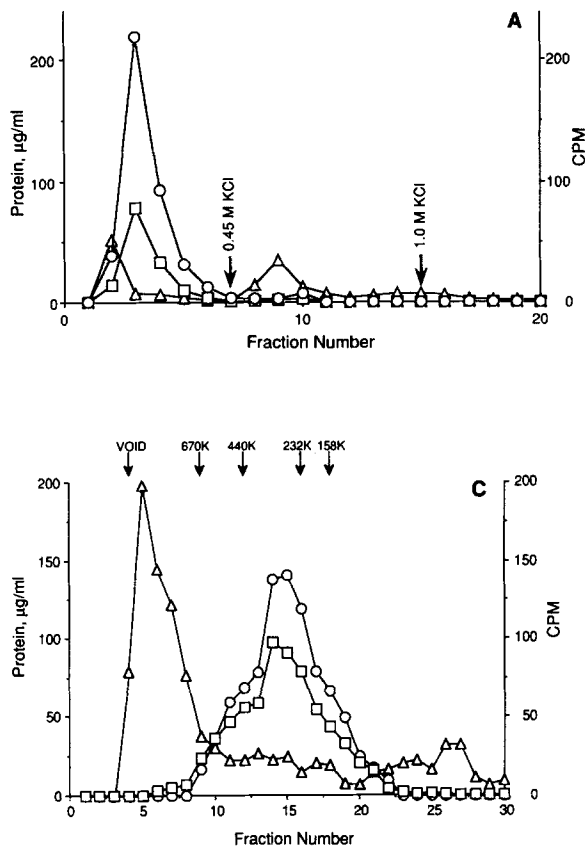


Figure 2. Cofractionation of L67 kinase and H1 kinase throughout several purification steps. (A) DEAE cellulose (DE-52). The column (1.5 × 2.5 cm) was equilibrated with buffer D containing 1 mM ATP and the material was eluted stepwise with buffer D/1 mM ATP containing different concentrations of KCl. (B) Phosphocellulose (p-11). The column (1.5 × 2.5 cm) was equilibrated with buffer D/1 mM ATP and eluted with a linear gradient of KCl in buffer D/1 mM ATP (broken line). (C) Gel filtration on Sephacryl S-300. The column (45 × 1.6 cm) was equilibrated and eluted with buffer D/1 mM ATP. Before analysis, 100 µl aliquots of the fractions collected in all procedures were passed through 1-ml spin-dry Sephadex G-25 columns equilibrated with buffer D/0.2% BSA (Materials and Methods). The positions of the molecular mass markers aldolase (158 kD), catalase (232 kD), ferritin (440 kD), and thyroglobulin (669 kD) are shown above the S-300 elution profile. (Δ—Δ) total protein, µg/ml (µg/ml × 10⁻¹ in Fig. 2 A); (□—□) H1 phosphorylation, cpm × 10⁻³; (○—○) L67 phosphorylation, cpm; (---) KCl gradient.

as a peak with an apparent molecular mass of ~80 kD (Fig. 3 A). A higher sedimentation rate (corresponding to an average of 200 kD) and a slightly more heterogeneous distribution were observed when KCl and CHAPS were omitted (not shown).

In all of these procedures, a single peak of NLD/L67-kinase activity was obtained coincident with that of histone H1 kinase (Figs. 2 and 3), and containing p34^{cdc2} and cyclin B (Fig. 3). These results suggested a stable physical association between NLD/L67-kinase activity and the p34^{cdc2}/cyclin B complex. The activity also caused a noticeable condensation of the chromosomes in isolated nuclei (Fig. 3 B; see also Dessev et al., 1989), similar to that observed in living clam oocytes after fertilization (not shown).

In the initial fractionation steps, the activity appeared to be associated with high molecular mass aggregates (Fig. 2 C). Treatment with CHAPS in the presence of 1 M KCl released an 80-kD complex by dispersing these aggregates without affecting the activity.

Further experiments revealed that in L67 modified by the

purified L67 kinase preparation the same major peptides 1 and 2, phosphorylated in vivo or in vitro with crude extracts, were labeled (Fig. 1, C compare with Fig. 1, A and B). This was also observed when, instead of isolated nuclei, L67 immunoadsorbed on protein A-Sepharose beads was used as a substrate (compare Fig. 1, C and G). The immunoadsorption procedure that involves boiling in SDS should destroy the secondary structure of L67 and irreversibly inactivate any protein kinase present in the preparation. The results with this substrate (Fig. 1, G and H; see also Fig. 3 C) suggest, therefore, that the specificity of the lamin kinase is determined primarily by the amino acid sequence of L67 rather than by its conformation. They also rule out the possibility that NLD and L67 phosphorylation involve a latent protein kinase associated with the nuclei and activated by p34^{cdc2}/cyclin B.

With the crude extract (Fig. 1 B), some of the spots observed with purified enzyme were absent (Fig. 1, C and G). This difference may be related to a number of factors. (a) In crude extracts the activity is in the form of large aggregates,

Figure 1. Two-dimensional tryptic phosphopeptide maps of L67 phosphorylated in vivo (A), and in vitro using NP-40 nuclei (B–F) or L67 immunoadsorbed on protein A-Sepharose beads (G and H) (autoradiographs). L67 was recovered from: (A) the soluble fraction after NEBD in vivo; (B) the soluble fraction after NLD in vitro in a crude extract from activated oocytes (60% solubilization of L67); (C) the soluble fraction after phosphorylation using clam oocyte p34^{cdc2}/cyclin B (sucrose gradient fractions No. 13–15; see Fig. 3 A); (D) the insoluble fraction after NLD and phosphorylation in a crude extract from activated oocytes (60% solubilization of L67); (E) the soluble fraction after phosphorylation with HeLa p34^{cdc2}/p62 (50% solubilization of L67); (F) the insoluble fraction after phosphorylation with HeLa p34^{cdc2}/p62 (50% solubilization of L67); (G) protein A-Sepharose after phosphorylation in the immunoadsorbed state using clam p34^{cdc2}/cyclin B (sucrose gradient fractions No. 13–15; see Fig. 3 A), and (H) Protein A-Sepharose beads after phosphorylation in the immunoadsorbed state using HeLa p34^{cdc2}/p62.

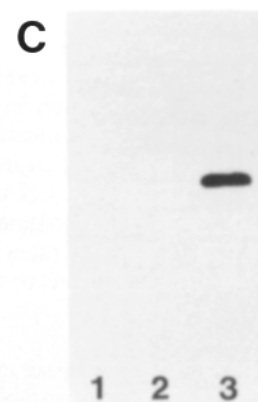
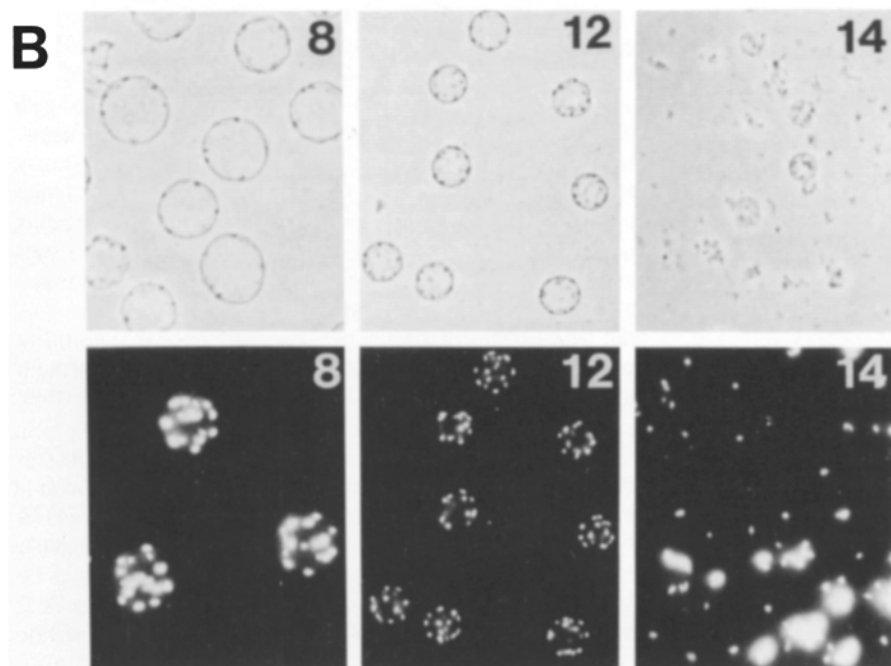
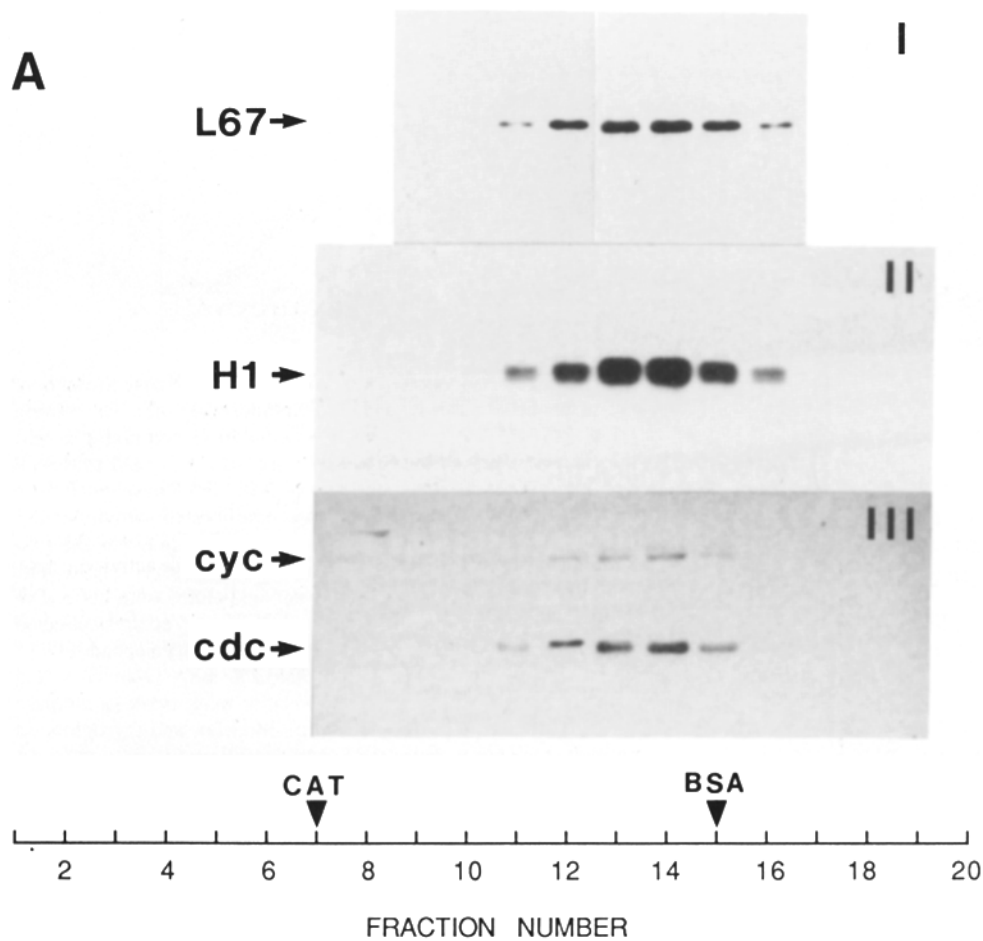


Figure 3. (A) Sedimentation profile of NLD/L67 kinase activity in a sucrose density gradient. 5 ml 5–20% linear sucrose gradients were prepared in buffer D containing 0.5 mM ATP, 1 M KCl, 0.5% CHAPS, and 0.2% BSA. Fractions No. 11–18 from the S-300 column (Fig. 2 C) were pooled and concentrated. 300 μ l aliquots of this material containing 1 M KCl, 1% CHAPS, and 0.2% BSA were applied to the gradients and centrifuged as described in Materials and Methods. The positions of the relative molecular mass markers (BSA, 67 kD; catalase, 232 kD) are shown. The fractions were passed through spin-dry Sephadex G-25 columns equilibrated with buffer D/0.2% BSA and analyzed as described in the text. (I) 32 P-labeled soluble L67 (autoradiograph); (II) histone H1-kinase activity (autoradiograph); (III)

while in the purified preparation it is associated with an 80-kD material. These two forms may have different accessibility to L67 for steric reasons, resulting in different phosphorylation patterns. (b) We have found that crude extracts from activated oocytes contain a protein phosphatase activity which is capable of dephosphorylating L67 (Dessev, G., unpublished data). This activity, which has not been detected in the preparations of partially purified L67 kinase, may preferentially remove phosphate from some of the sites of L67 after its solubilization, thus accounting for the absence of some spots from the map in Fig. 1 B (compare with Fig. 1 D). (c) The differences in the relative intensities of the spots may be due to changes of the kinase activity (partial inactivation) during the incubation (our experiments do not distinguish between phosphorylation of L67 before and after its solubilization). Although we are unable to distinguish among these explanations at the present time, it appears from our results that the modification of the two major sites corresponding to phosphopeptides 1 and 2 are essential for NLD, while that of the additional sites may not be necessary.

NLD/L67 Kinase Activity Is Depleted by Treatment with p13-Sephacrose Beads and Is Restored by HeLa p34cdc2/p62 Complex

The results described above suggest that a p34^{cdc2}/cyclin B complex is directly involved in L67 phosphorylation. To examine this possibility further, the active fractions from the sucrose gradient (No. 13–15; see Fig. 3) were pooled, passed through a spin-dry Sephadex G25 column equilibrated with buffer D containing 0.2% BSA and 0.5 mM ATP, and then supplied with an ATP regenerating system. Aliquots of this sample were then treated with Sepharose beads conjugated to p13^{suc1} (p13-Sephacrose beads). p13 is a yeast protein that binds p34^{cdc2} specifically to form a stable complex (Brizuela et al., 1987; Dunphy and Newport, 1989). Control samples were exposed to Sepharose conjugated to BSA. Bound and unbound materials were then characterized.

Both p34^{cdc2} and cyclin B were retained on p13-Sephacrose beads and were not detectable in the solution by immunoblotting (Fig. 4, lanes 1–4). The only polypeptide bound to the p13-Sephacrose beads, which was phosphorylated under the conditions of the protein kinase assay, had an electrophoretic mobility identical to that of cyclin B (Fig. 4, lane 5). Incubation in the presence of [γ -³²P]ATP and histone H1 revealed that most of the H1-kinase activity was also depleted from the extract (Fig. 4, lanes 6 and 7). No p34^{cdc2} or cyclin B were found associated with the control BSA-Sephacrose beads (data not shown). These results are in agreement with the findings of Draetta et al. (1989) who have shown that both cyclin A and B form complexes with p34^{cdc2} that are retained on p13-Sephacrose beads, and that cyclins are in vitro substrates for p34^{cdc2} protein kinase. We have not assayed for cyclin A since it is not present in oocytes (Swenson et al., 1986).

The treatment with p13-Sephacrose beads depleted the

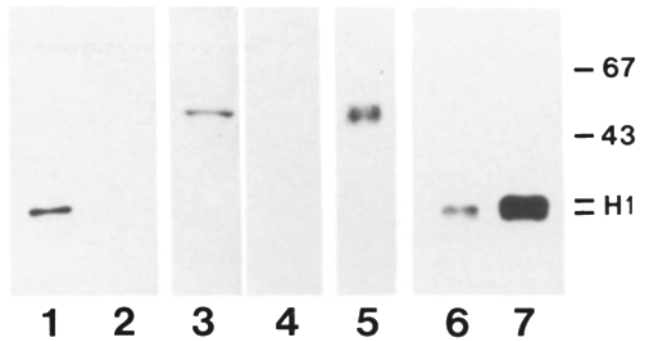


Figure 4. The fractions from the sucrose gradient shown in Fig. 3 containing the highest NLD activity (No. 13–15) were pooled and treated with p13-Sephacrose as described in Materials and Methods. The solution and the beads were analyzed separately. Lane 1, p34^{cdc2} eluted from the beads; lane 2, p34^{cdc2} in solution; lane 3, cyclin B eluted from the beads; lane 4, cyclin B in solution; lane 5, beads were incubated with [γ -³²P]ATP, and the retained material was eluted and analyzed; lane 6, H1 kinase activity in samples treated with p13-Sephacrose; lane 7, H1-kinase activity in the control sample treated with BSA-Sephacrose. Lanes 1–4 represent immunoblots, and lanes 5–7 represent autoradiographs. The positions of relative molecular mass markers are shown.

pooled gradient peak fractions of NLD/L67 kinase activity. In the control samples treated with BSA-Sephacrose beads the nuclei were completely disassembled in 30 min (Fig. 5 A, panel 1) and virtually all of L67 was found in the supernatant in the phosphorylated state (Fig. 5 B, lane 1). Solubilization of L67 was also evident by immunoblotting (data not shown). In the sample treated with p13-Sephacrose beads the nuclei remained morphologically unaltered (Fig. 5 A, panel 2), and the level of L67-kinase was decreased to 15% of the control, as determined by measuring the radioactivity in the L67 gel fraction (Fig. 5 B, lane 2).

To test for the existence of a lamin kinase different from p34^{cdc2}/cyclin B, crude extracts and also the fraction precipitated at 33% ammonium sulfate saturation were treated with p13-Sephacrose beads. In all cases we observed a strong decrease in L67 kinase activity and a loss of NLD activity (not shown; results similar to those in Fig. 5). The depleted samples contained a strong protein kinase activity which phosphorylated a number of endogenous proteins, but not L67 (not shown). These results suggest that in the clam oocytes the lamin phosphorylation is catalysed by a single protein kinase containing p34^{cdc2}.

To further investigate the role of p34^{cdc2} and cyclin B in the process of NLD, we added to the depleted samples p34^{cdc2} kinase purified from HeLa cells which is associated with a p62 subunit, a cyclin B-like molecule (Brizuela et al., 1989; Solomon et al., 1988). After this treatment, NLD activity was restored (Fig. 5 A, panel 3), in parallel with an increase in the L67 phosphorylation corresponding to 78% of the control level (Fig. 5 B, lane 3). These results provide

distribution of p34^{cdc2} and cyclin B examined using a mixture of the corresponding antibodies (immunoblot). (B) Morphological changes in the nuclei incubated for 30 min in the presence of gradient fractions under standard conditions for NLD in vitro. (Top) Phase contrast; (bottom) fluorescence (Hoechst 33342). The numbers on the micrographs correspond to the numbers of the gradient fractions. Note that the chromosomes become more compact as the nuclei approach disassembly. (C) Phosphorylation of L67 immunoadsorbed on protein A-Sephacrose beads and incubated in a standard protein kinase reaction mixture in the presence of buffer D (lane 1), gradient fraction No. 5 (lane 2), and pooled gradient fractions No. 13–15 (lane 3).

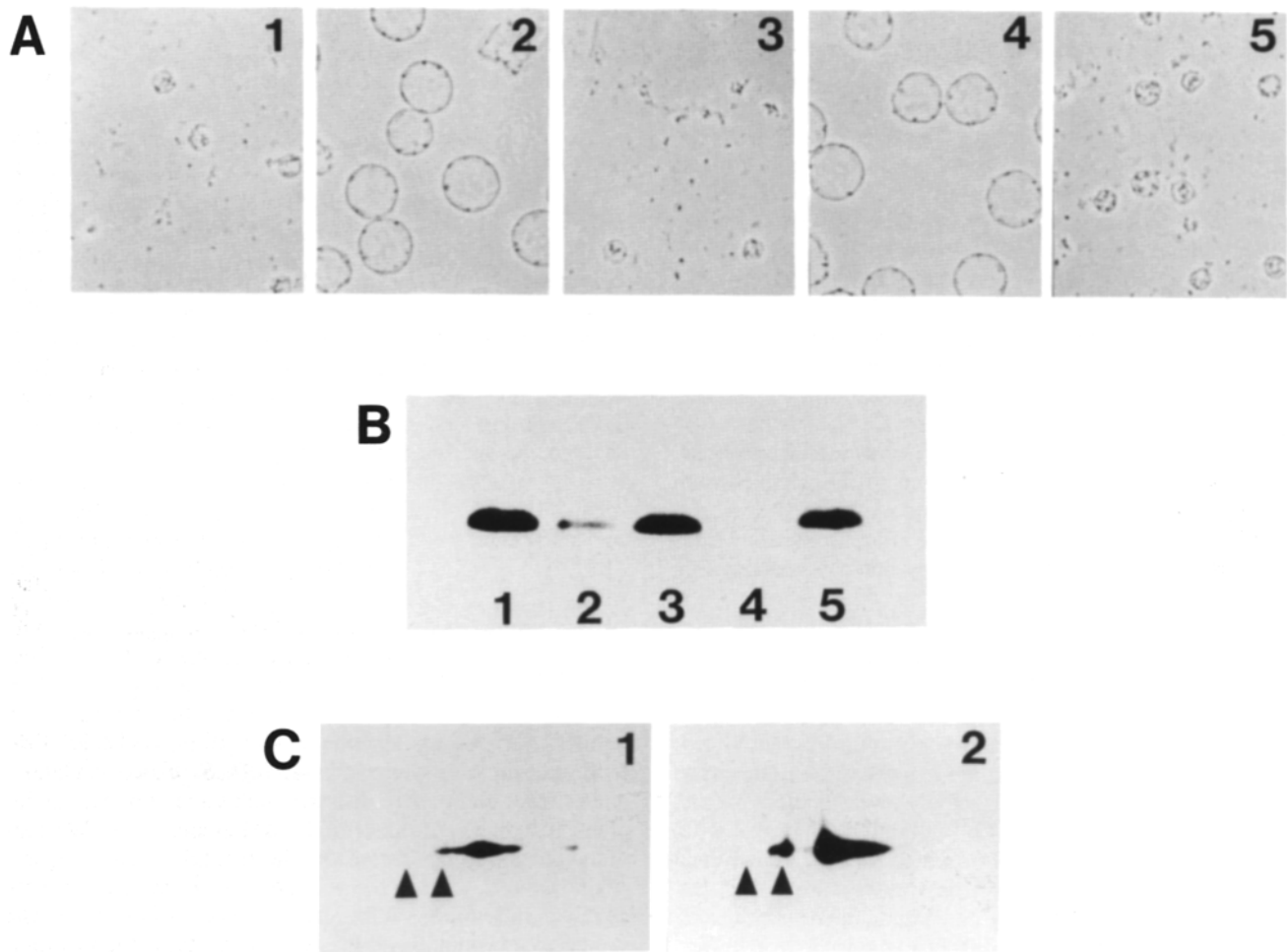


Figure 5. Effect of p13-Sepharose and p34^{cdc2}/cyclin B complex from HeLa cells on L67 phosphorylation and solubilization. Pooled active gradient fractions (No. 13–15 in Fig. 3 A) were treated with BSA-Sepharose (A, panel 1; B, lane 1), p13-Sepharose (A, panel 2; B, lane 2), and p13-Sepharose with subsequent addition of 2.5 μ l of HeLa p34^{cdc2}/p62 preparation per 20 μ l (A, panel 3; B, lane 3). After removal of the beads, 20 μ l aliquots of the solutions were mixed with $\sim 5 \times 10^3$ oocyte nuclei and incubated for 30 min in the presence of 0.5 mM [γ -³²P]ATP. The samples were then centrifuged for 5 min at 14,000 rpm, L67 was recovered from the supernatants by immunoprecipitation, and analyzed by SDS-PAGE and autoradiography. The same amount of nuclei was incubated for 90 min in buffer D/0.2% BSA alone (A, panel 4; B, lane 4), and in buffer D/0.2% BSA containing 2.5 μ l of HeLa p34^{cdc2}/p62 per 20 μ l (A, panel 5; B, lane 5). The soluble L67 was immunoprecipitated and analyzed in the same way. (A) Phase-contrast micrographs of nuclei incubated in the corresponding samples for 30 (1, 2, and 3) and 90 min (4 and 5). (B) Autoradiographs of L67 immunoprecipitated from the corresponding soluble fractions. The L67 gel fractions contained 184, 29, 144, 14, and 120 Cerenkov cpm for lanes 1–5, respectively. (C) Two-dimensional IEF/SDS-PAGE of L67 after phosphorylation using pooled active gradient fractions (Fig. 3 A) (panel 1) or HeLa p34^{cdc2}/p62 (panel 2). L67 was recovered from the soluble fractions and fractionated in the presence of unlabeled L67 isolated from intact nuclei. The soluble radioactive L67 (autoradiographs) is shifted to the right towards the acid (+) pole of the IEF gel, relative to the positions of the two main isoelectric variants of the polymeric L67, determined by staining with Coomassie blue (arrowheads).

further evidence that NLD/L67 kinase activity in our preparations is the p34^{cdc2}/cyclin B complex.

HeLa p34^{cdc2}/p62 Alone Phosphorylates L67 and Causes Disassembly of Detergent-purified Nuclei

Further evidence for the direct role of p34^{cdc2}/cyclin B in L67 phosphorylation and NLD was obtained in the experiment shown in Fig. 5. NP-40 nuclei were incubated at room temperature in buffer D/0.2% BSA in the presence of 0.5 mM [γ -³²P]ATP and the ATP-regenerating system, with or without addition of HeLa p34^{cdc2}/p62. After 90 min, the nuclei in the control sample were unchanged (Fig. 5 A, panel 4) and no radioactive L67 was released in the solu-

ble fraction (Fig. 5 B, lane 4). During the same period of time, the nuclei in the sample containing HeLa p34^{cdc2}/p62 underwent the changes characteristic for nuclear disassembly (Dessev et al., 1989), i.e., their size was reduced and they appeared mechanically destabilized. After 90 min, >70% of the nuclei in this sample were completely disassembled, the remaining nuclei being in an advanced state of disassembly (Fig. 5 A, panel 5), and radioactive L67 (65% of the control level) was found in the supernatant (Fig. 5 B, lane 5). These results demonstrate that the heterologous HeLa p34^{cdc2}/p62 alone is capable of phosphorylating L67 and disassembling oocyte nuclei in vitro.

To show that the p34^{cdc2}/p62-dependent incorporation of

^{32}P reflected an increase in the phosphate content of L67 and not a higher rate of phosphate turnover, we examined the behavior of L67 in 2D IEF/SDS gels. L67 phosphorylated and solubilized by both clam NLD/L67 kinase activity and HeLa p34^{cdc2}/p62 was displaced by about 0.5 pH units towards the acid (+) pole of the gel, relative to the bulk L67 isolated from intact nuclei (Fig. 5 C). A shift of a similar magnitude has been noticed earlier both in vivo and in vitro and has been shown to correspond to an increase with 2.6–3.2 phosphoryl residues per L67 molecule (Dessev and Goldman, 1988; Dessev et al., 1989). These results demonstrate that solubilization of L67 by both the homologous NLD/L67 kinase activity and the heterologous p34^{cdc2}/p62 is paralleled by a similar increase in the phosphate content of this protein.

We further examined the specificity of the HeLa p34^{cdc2}/p62 kinase by phosphopeptide mapping. The maps obtained using both NP-40 nuclei (Fig. 1, E and F), and L67 immunoadsorbed onto protein A-Sepharose (Fig. 1 H) are similar to the maps of L67 modified by the homologous enzyme. Phosphopeptide 2 is seen only in the soluble form of L67 (Fig. 1 E) but not in the "polymeric" one (Fig. 1 F), emphasizing its importance for NLD. On the basis of these results it can be concluded that the primary specificities of the homologous and heterologous enzyme are very similar.

In contrast to the above, we have observed no significant lamin phosphorylation (<5% of the phosphorylation level of the L67 solubilized in vitro by the endogenous activity, with no shift in pI) and no nuclear disassembly, upon treatment of oocyte nuclei with a large amount (250 U) of the catalytic subunit of the cAMP-dependent protein kinase (data not shown).

Discussion

Shortly after fertilization, MPF activity appears in the clam oocytes, as established by the microinjection assay (Kishimoto et al., 1984). Coincident with MPF activation, the oocytes undergo NEBD (Allen, 1953) accompanied by an increase in the level of L67 phosphorylation of 2–3 mol phosphate per mole protein (Dessev and Goldman, 1988). In fertilized clam oocytes, the presence of a complex containing p34^{cdc2} and cyclin B has been demonstrated, and the level of its functional equivalent, H1 kinase, has been found to display cell cycle-dependent variations, characteristic for MPF activity (Draetta et al., 1989). A number of studies have provided convincing evidence that p34^{cdc2} and cyclins are major components of MPF (Dunphy et al., 1988; Gautier et al., 1988; Labbe et al., 1989; Gautier et al., 1990).

In this work we show that in the clam oocytes there is a single lamin-specific protein kinase which represents a complex containing p34^{cdc2} and cyclin B and is directly involved in NLD in clam oocytes. This conclusion is based (a) on the finding that both L67 kinase activity and NLD activity cofractionate with p34^{cdc2}/cyclin B and H1 kinase activity through a number of purification procedures, (b) on the observation that NLD activity is depleted by p13-Sepharose beads and is restored by the addition of a highly purified p34^{cdc2}/p62 kinase from HeLa cells, (c) on the ability of HeLa p34^{cdc2}/p62 alone to phosphorylate L67 and induce NLD, and (d) on the similarity between the phosphopeptide maps of L67 modified in vivo and in vitro, in the latter case

by both clam p34^{cdc2}/cyclin B and the analogous complex from HeLa cells. This conclusion is in agreement with the recent results of Peter et al. (1990).

In crude extracts the majority of NLD/L67 kinase activity is associated with a high speed sedimentable fraction (Dessev, G., unpublished data). Similarly, during gel filtration on Sephacryl S-300, the activity behaves as 200–400-kD material. At present, the role of these complexes is not clear, but further analysis may reveal other functionally important components complexed to p34^{cdc2}/cyclin B. In sucrose gradients containing CHAPS and 1 M KCl, the size of the active complex is reduced to an apparent M_r of ~80 kD, which is consistent with a heterodimer of one molecule of p34^{cdc2} and one molecule of cyclin B, as demonstrated recently for MPF in other systems (Lohka et al., 1988; Labbe et al., 1989; Erikson and Maller, 1989).

In contrast to other cell-free systems developed for studying the M-phase events (Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Newport, 1987; Newport and Span, 1987; Nakagawa et al., 1989), the present results have been obtained with a homologous system, i.e., nuclei as well as extracts prepared from the same type of cells are used both to reproduce NLD in vitro and as a source for the purification of NLD/L67 kinase activity. The morphological and biochemical aspects of NEBD of clam oocytes in vivo and in vitro, including chromosome condensation, L67 solubilization, and the level and specificity of L67 phosphorylation, are very similar, as demonstrated in this and previous studies (Dessev and Goldman, 1988; Dessev et al., 1989). We consider it very likely, therefore, that a single protein kinase, which appears to be identical to MPF, is the enzyme directly involved in lamin phosphorylation and NEBD in vivo. The data suggest that the modification of two sites in the L67 molecule, located in phosphopeptides 1 and 2 (Fig. 1), is involved in the M-phase transition of L67 from a polymeric to a soluble state, in agreement with Heald and McKeon (1990) and Ward and Kirschner (1990). More definite conclusions on this point will have to await the sequencing of L67 and localization of its phosphorylation sites.

While in the system of Peter et al. (1990) no changes in the degree of chromatin condensation take place, the present experiments suggest that the p34^{cdc2}-H1 kinase may be directly involved in the cell cycle-dependent changes in chromatin condensation. However, it should be taken into consideration that the oocyte chromosomes are meiotic tetrads which are partially condensed. It remains to be determined whether MPF is directly involved in chromatin condensation during mitosis.

It is interesting to note that in our experiments L67 phosphorylation and NLD can be achieved in vitro by two protein kinase preparations in which the catalytic subunit p34^{cdc2} is associated with different, presumably regulatory, subunits (clam cyclin B and HeLa p62). In this regard, cyclins have been postulated to assign different specificities to p34^{cdc2} (Draetta et al., 1989; for discussion see Lohka, 1989; Nurse, 1990). However, such differences may be lost or difficult to observe in vitro.

Previous studies with *Xenopus* have failed to show lamins as substrates for p34^{cdc2}, perhaps due to the origin and the structural state of the lamins used in the assays (Lohka et al., 1988). This led to the assumption that the lamin kinase is different from MPF (Lohka et al., 1988). Miake-Lye and

Kirschner (1985) and Newport and Spann (1987) have reached a similar conclusion based on the existence of a lag between the addition of MPF to their cell-free extracts and the onset of lamin phosphorylation. The reasons for these differences are not clear but could be related to additional processes occurring in crude cell-free systems.

The existing assays for MPF activity *in vivo* (microinjection) and *in vitro* (crude cell-free extracts) involve extremely complex and technically difficult systems (Masui and Markert, 1971; Lohka and Maller, 1985; Murray and Kirschner, 1989b), with inherent limitations in studying the biochemistry of MPF. Our results suggest a much simpler, direct, functional test for MPF activity that uses both morphological and biochemical criteria in a single cell type. The *Spisula* oocyte system should be useful for investigating the structural and biochemical aspects of NEBD, and the regulation of MPF activity.

Finally, so far MPF has been viewed as the main controlling element of the cell cycle that acts at the beginning of the pathways leading to the M-phase specific events. In the light of our results (see also Peter et al., 1990) it appears more likely that MPF, being directly involved in M-phase-specific reactions at least in some cases, operates at the "executive" level. This emphasizes the importance of as yet unknown elements in the regulation of the cell cycle operating upstream of MPF.

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