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A cyclin-abundance cycle-independent p34^{cdc2} tyrosine phosphorylation cycle in early sea urchin embryos

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The activity of the cell cycle control protein p34^{cdc2} is post-translationally regulated in a variety of cell types. Using anti-phosphotyrosine antibodies, we find that p34^{cdc2}-directed tyrosine kinase activity increases at fertilization in sea urchin eggs, leading to a gradual accumulation of phosphotyrosine on p34 during the early part of the cell cycle. Loss of phosphotyrosine from p34 accompanies entry into mitosis and phosphotyrosine re-accumulates as the embryo enters the next cell cycle. A similar pattern is seen when eggs are parthenogenetically activated with ammonium chloride. Tyrosine phosphorylation and phosphorylation/dephosphorylation cycles are suppressed when embryos are treated with the tyrosine kinase inhibitor genistein. On the other hand, a cycle persists when protein synthesis is inhibited with emetine, indicating that it is independent of the synthesis of another class of cell cycle control proteins, the cyclins. Additional experiments with the phorbol ester, phorbol myristate acetate, demonstrate that activating protein synthesis alone in unfertilized eggs does not result in stimulation of p34^{cdc2} tyrosine kinase activity. Our results indicate that p34 tyrosine phosphorylation cycles are triggered by the fertilization Ca_i transient. The first cycle is independent of the fertilization pH_i signal, confirming that, in sea urchin embryos, the cycle is not tightly coupled to the cycle of cyclin abundance that is a prominent feature of the eukaryotic cell division cycle.

Key words: cell cycle/cyclin/mitosis/phosphorylation/sea urchin

Introduction

The cell division cycle in early sea urchin embryos comprises several interlocking sub-cycles: the centrosome duplication cycle (Mazia *et al.*, 1960; Sluder and Lewis, 1987); a cyclic alteration in the properties of the cortical cytoskeleton (Swann and Mitchison, 1953; Yoneda *et al.*, 1978); the nuclear cycle of S-phase and mitosis (Mazia, 1974; Mazia and Ruby, 1974); the cycle of kinase activity associated with mitosis (Meijer and Pondaven, 1988; Arion and Meijer, 1989); and the cyclic accumulation and destruction of the cyclins, prominent cell cycle control proteins (Evans *et al.*, 1983). Mitosis and mitotic kinase activity are suppressed by inhibitors of protein synthesis (Wagenaar and Mazia, 1978; Meijer and Pondaven, 1988), suggesting that cyclin (or another protein newly synthesized at fertilization) is at least in part responsible for activation of the mitotic kinase and entry into mitosis. In contrast, the centrosome duplication cycle and S-phase are independent of protein synthesis (Wagenaar and Mazia, 1978; Sluder *et al.*, 1990), indicating that they are driven by other mechanisms than the cyclin-abundance cycle. Here, we describe a sub-cycle that comprises the cyclic tyrosine phosphorylation of the cell cycle control protein p34^{cdc2} and test its dependency on the sub-cycle of cyclin abundance.

p34^{cdc2} is a well-conserved and widely distributed protein that is essential for mitosis entry (Nurse, 1990). It is one component of the mitotic kinase that drives the nucleus into mitosis. The levels of p34 do not alter during the cell cycle in yeast (Lee and Nurse, 1987), Xenopus and starfish oocytes (Labbe *et al.*, 1988a,b), surf clam eggs (Draetta *et al.*, 1989), sea urchin embryos (Meijer *et al.*, 1989) or mammalian cells (Lee *et al.*, 1988), but the protein is differentially phosphorylated at different cell cycle stages (Draetta and Beach, 1988; Draetta *et al.*, 1988; Lee *et al.*, 1988; Gautier *et al.*, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989; Nurse, 1990). Activation of the mitotic kinase activity is associated with a marked and rapid dephosphorylation of p34 phosphotyrosine (Gould and Nurse, 1989; Morla *et al.*, 1989; Pondaven *et al.*, 1990). In yeast, phosphorylation of tyrosine 15 may maintain the kinase in an inactive state, since a mutant in which tyrosine 15 is replaced with phenylalanine has the *wee* phenotype that accompanies precocious entry into mitosis (Gould and Nurse, 1989). The other component of the mitotic kinase is a cyclin (Labbe *et al.*, 1989; Meijer *et al.*, 1989; Gautier *et al.*, 1990; Minshull *et al.*, 1990). In cell free systems (Murray and Kirschner, 1989; Murray *et al.*, 1989; Luca and Ruderman, 1989) and in *Xenopus* oocytes (Swenson *et al.*, 1986) cyclin accumulation or cyclin microinjection triggers entry into M-phase and in *Xenopus* embryos, specifically interfering with cyclin expression using antisense oligonucleotides prevents mitosis (Minshull *et al.*, 1989). The activity of the mitotic kinase in sea urchin eggs correlates well with cyclin levels (Meijer *et al.*, 1989). These observations suggest at least two levels of control for the mitotic kinase, one involving dephosphorylation of tyrosine 15 on p34 and the other the abundance of cyclin. Are the two levels of control independent, or does cyclin abundance control the tyrosine phosphorylation state of p34? Our results indicate that the two controls are distinct, though loosely coupled.

Results

Tyrosine phosphorylation of p34 during the cell cycle in sea urchin embryos

Unfertilized sea urchin eggs are arrested in interphase. The embryo enters S-phase 30 min after fertilization and mitosis occurs 30 min later. Figure 1 shows an experiment in which phosphotyrosine phosphorylation of p34^{cdc2} was measured

using an anti-phosphotyrosine antibody (Kamps and Sefton, 1988). The unfertilized egg contains low levels of phosphotyrosine-containing p34. Phosphotyrosine accumulates gradually on p34 after fertilization and reaches a peak 50 min into the first cell cycle. The subsequent decrease in phosphotyrosine levels correlates with entry into mitosis [nuclear envelope breakdown (NEB)]. During mitosis, phosphotyrosine levels are lower than those measured in the unfertilized egg. As the nucleus leaves mitosis, at 90 min, phosphotyrosine again accumulates on p34; relative to the unfertilized egg, the increase is smaller than in the first cell cycle, but relative to first mitosis, the increase is comparable, ~5-fold. These data indicate that a p34-tyrosine phosphorylation/dephosphorylation cycle accompanies the mitotic cycle in sea urchin eggs, mitosis being correlated with loss of phosphotyrosine from p34.

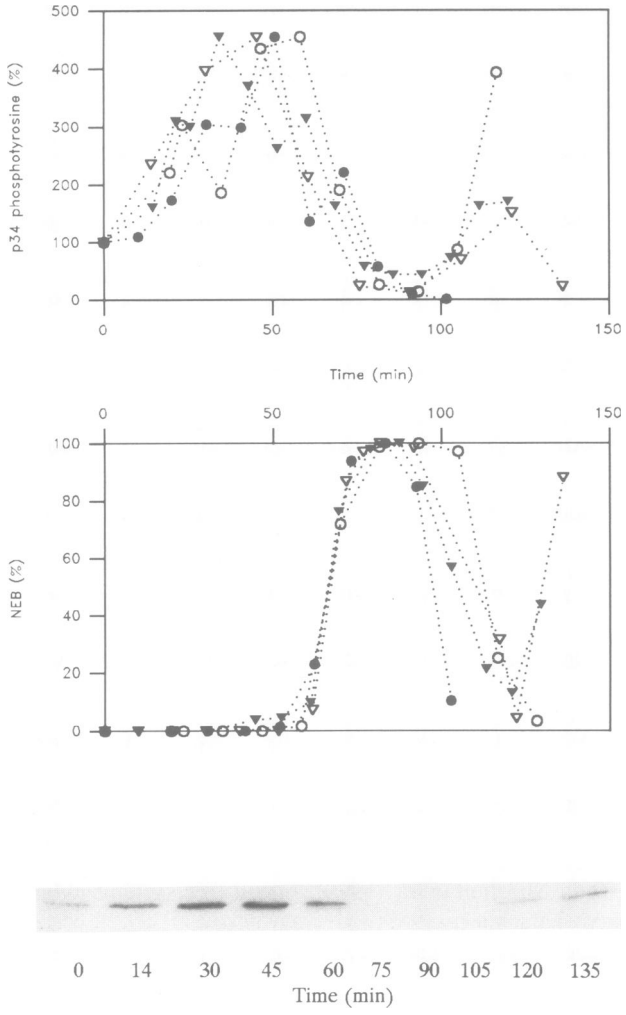

Fig. 1. p34 phosphotyrosine levels after fertilization, measured from Western blots using an antiphosphotyrosine antibody. p34 was extracted from embryo homogenates at various times after fertilization using p13-conjugated Sepharose beads and resolved using PAGE. **Top**: Quantification (see Materials and methods) of phosphotyrosine levels in four experiments and correlation with nuclear envelope breakdown. The results are expressed in terms of the phosphotyrosine content of p34 in the unfertilized egg; the data were normalized to the peak phosphotyrosine level [454 ± 65% (mean and SEM, $n = 4$)]. Time to 50% NEB ranged from 63–74 min [67.3 ± 2.3 (mean and SEM)] and was normalized to the mean. **Bottom**: Autoradiogram of a Western blot using an anti-phosphotyrosine antibody. *L.pictus* 16°C.

Inspection of the anti-phosphotyrosine antibody blots indicates that two closely migrating bands crossreact with the antibody in unfertilized eggs (Figure 2). Immediately after fertilization, the accumulating crossreactivity is confined to the upper of the two bands. Later, just prior to the disappearance of crossreactivity, a doublet is again seen. Closely migrating bands are also seen when blots are treated with the anti-p34 PSTAIR antibody (not shown). These observations suggest either that multiple isoforms of p34 exist that show opposite patterns of tyrosine phosphorylation or that the tyrosine phosphorylation that we measure is predominantly associated with serine-or threonine-phosphorylated p34.

p34 tyrosine phosphorylation and dephosphorylation are triggered by ammonium chloride, a parthenogenetic activator

Treating unfertilized eggs briefly with ammonium chloride activates the nuclear duplication cycle. Ammonium chloride-treated eggs undergo repeated rounds of DNA synthesis, nuclear envelope breakdown and reformation and chromatin condensation (Mazia, 1974; Mazia and Ruby, 1974), albeit more slowly than fertilized embryos. The mitotic and cleavage apparatus are absent in parthenogenetically activated eggs, since they lack the centrosome organizer that derives from the sperm in a fertilized egg (Brandriff *et al.*, 1975). Treatment with ammonia leads to activation of the mitotic kinase, co-incident with mitosis (Meijer and Pondaven, 1988). It also triggers accumulation of phosphotyrosine on p34 (Figure 3). Nuclear envelope breakdown and chromatin condensation are accompanied by loss of the phosphotyrosine. These data confirm the inverse correlation between p34 phosphotyrosine levels and NEB/chromatin condensation and indicate that the phosphotyrosine cycle does not require a functioning mitotic apparatus.

The tyrosine kinase inhibitor genistein suppresses p34 phosphorylation and blocks mitosis

Genistein is an isoflavone that inhibits tyrosine kinases by competing at the ATP binding site (Akiyama *et al.*, 1987). Incubating embryos in sea water containing 100 μM genistein suppresses tyrosine phosphorylation of p34 ~5-fold and prevents mitosis (Figure 4A). Lower concentrations of genistein delay mitosis (Figure 4B). These data suggest that the levels of p34 phosphotyrosine are a factor in determining the timing of mitosis entry, as in yeast (Gould and Nurse,

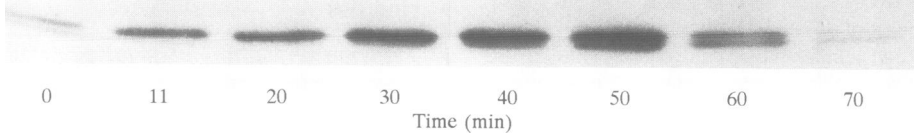

Fig. 2. The anti-phosphotyrosine antibody detects two closely migrating bands in p13 extracts that cross-react with an anti-p34 antibody (see Materials and methods) and alter in both absolute and relative intensity as the cell cycle progresses. Time after fertilization is shown. *L.pictus.* 16°C.

1989). However, whereas suppressing tyrosine phosphorylation of p34 by amino acid substitution in yeast advances mitosis entry, in sea urchin embryos treated with genistein, mitosis is retarded.

Inhibition of protein synthesis does not suppress the initial cycle of p34 tyrosine phosphorylation/ dephosphorylation

Emetine, an inhibitor of EF2-dependent polysome translocation (Vasquez, 1979), prevents protein synthesis in early sea urchin embryos (Wagenaar and Mazia, 1978). At a concentration of 100 μM in sea water, it inhibits incorporation of labelled amino acids into protein by more than 95% (Wagenaar and Mazia, 1978), prevents entry into mitosis (Wagenaar and Mazia, 1978) and suppresses activation of the mitotic kinase (Meijer and Pondaven, 1988). Figure 5 shows that a cycle of tyrosine phosphorylation and dephosphorylation of p34 persists in embryos incubated with this concentration of emetine. However, peak p34 phosphotyrosine is 70% of controls and it subsequently decreases more slowly than in control embryos. These data indicate that tyrosine phosphorylation of p34 is not tightly coupled to the cyclin abundance cycle, at least during the first cell cycle.

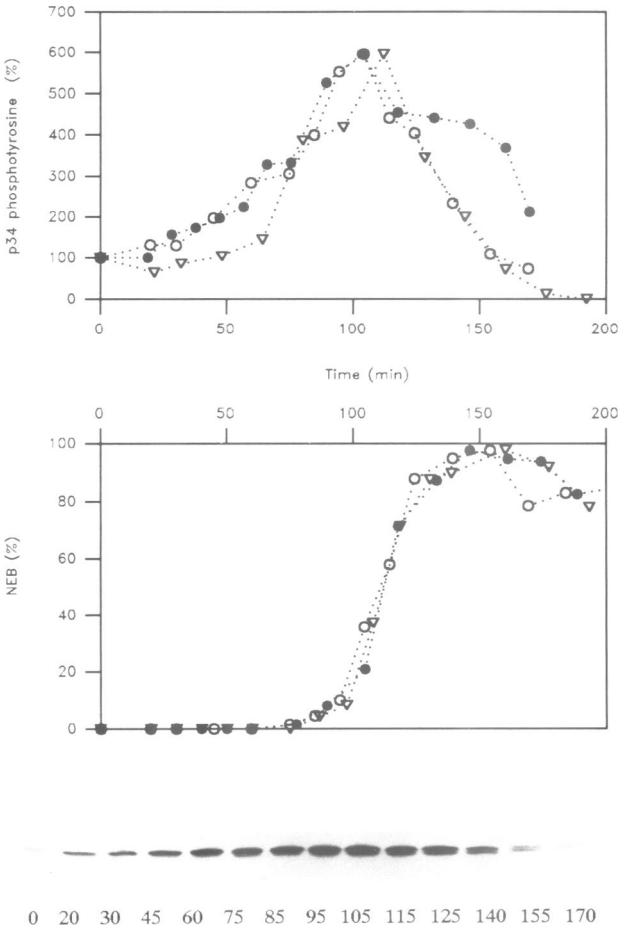

Fig. 3. p34 phosphotyrosine levels and nuclear envelope breakdown in ammonia-activated eggs. Unfertilized eggs were treated with 15 mM NH_4Cl in artificial seawater (ASW), pH 9.0 for 15 min. **Top**: Quantification of data from three experiments. The maximal increase in phosphotyrosine was 596 ± 122% [mean and SEM, n =3]. The normalization method is described in the legend to Figure 1. **Bottom**: Autoradiogram of one of the three experiments. *L.pictus*. 16°C.

Treatments that increase the intracellular pH of unfertilized eggs and stimulate cyclin synthesis do not lead to tyrosine phosphorylation of p34^{cdc2}

Treatment of unfertilized eggs with the phorbol ester, phorbol myristate acetate (PMA) causes an increase in intracellular pH (pH_i) by activating a sodium hydrogen antiporter that is regulated in part by protein kinase C (Swann and Whitaker, 1985; Shen and Buck, 1990). The rise in pH_i increases the rate of protein synthesis (Whitaker and Steinhardt, 1982; Swann and Whitaker, 1985) and stimulates cyclin accumulation to levels comparable to those measured in fertilized eggs (Whitaker and Patel, 1990). However, there is no detectable increase in p34 phosphotyrosine levels after treatment with PMA (Figure 6). Concentrations of ammonium chloride too low to cause chromatin condensation cycles but sufficient to increase pH_i and protein synthesis rates (Epel *et al.*, 1974) also fail to induce tyrosine phosphorylation of p34 (not shown). These data show that neither a pH_i increase nor cyclin synthesis (both of which occur in fertilized eggs immediately after fertilization) are sufficient to induce p34 tyrosine phosphorylation.

The p34^{cdc2} tyrosine phosphorylation cycle is controlled by the fertilization Ca_i signal

The interphase cell cycle arrest of unfertilized sea urchin eggs is reversed by a global increase in intracellular free calcium ion concentration (Ca_i) at fertilization (Whitaker and Steinhardt, 1982; Whitaker and Patel, 1990). The pH_i signal that governs cyclin synthesis is dependent on the Ca_i increase (Whitaker and Patel, 1990). We can study the effects of the Ca_i signal alone by fertilizing eggs in sodium-free sea water in the presence of the drug dimethylamiloride (Swann and Whitaker, 1985). These conditions inhibit the sodium−hydrogen antiporter and prevent the pH_i increase.

The p34 tyrosine phosphorylation cycle persists after fertilization in the absence of a pH_i increase (Figure 7). The cycle is slower than in control eggs and resembles the cycle we found in embryos treated with the protein synthesis inhibitor emetine. These data indicate that phosphotyrosine accumulation on p34^{cdc2} after fertilization is triggered by the Ca_i signal, not the pH_i signal, and confirm that the cycle is independent of protein synthesis.

Discussion

Stimulation of p34 tyrosine phosphorylation at fertilization

We find detectable levels of tyrosine phosphate in p34^{cdc2} in unfertilized eggs, using phosphotyrosine antibodies. Fertilization causes a marked increase in the rate of net p34 tyrosine phosphorylation, leading to a gradual accumulation of phosphotyrosine in p34. We do not know whether the net increase is due to activation of a tyrosine kinase, inactivation of a tyrosine phosphatase or a substrate modification of p34 that makes it more susceptible to phosphorylation by a constitutively-active tyrosine kinase. The last of these mechanisms suggests itself to us only because phosphotyrosine is found immediately after fertilization predominantly in the more slowly-migrating p34 band on polyacrylamide gels. This band may represent a highly phosphorylated form of p34.

The stimulus to the increased rate of p34 tyrosine phosphorylation is the fertilization Ca_i transient. The causal link is inevitable, in a trivial sense, since the calcium transient

is the necessary and sufficient stimulus for resumption of the cell cycle at fertilization (Whitaker and Steinhardt, 1982; Whitaker and Patel, 1990). Less trivially, we have shown that the dependent ionic signal at fertilization, the pH increase, is neither necessary nor sufficient to induce p34 tyrosine phosphorylation. We conclude that the increase in phosphotyrosine in p34 after fertilization is a direct consequence of the Ca_i signal and independent of pH_i. In this it resembles the increased ^{32}P-incorporation into p34 (Patel *et al.*, 1990; Whitaker and Patel, 1990). However, ^{32}P-incorporation after fertilization is rapid and precedes the gradual phosphorylation of tyrosine residues that we report here.

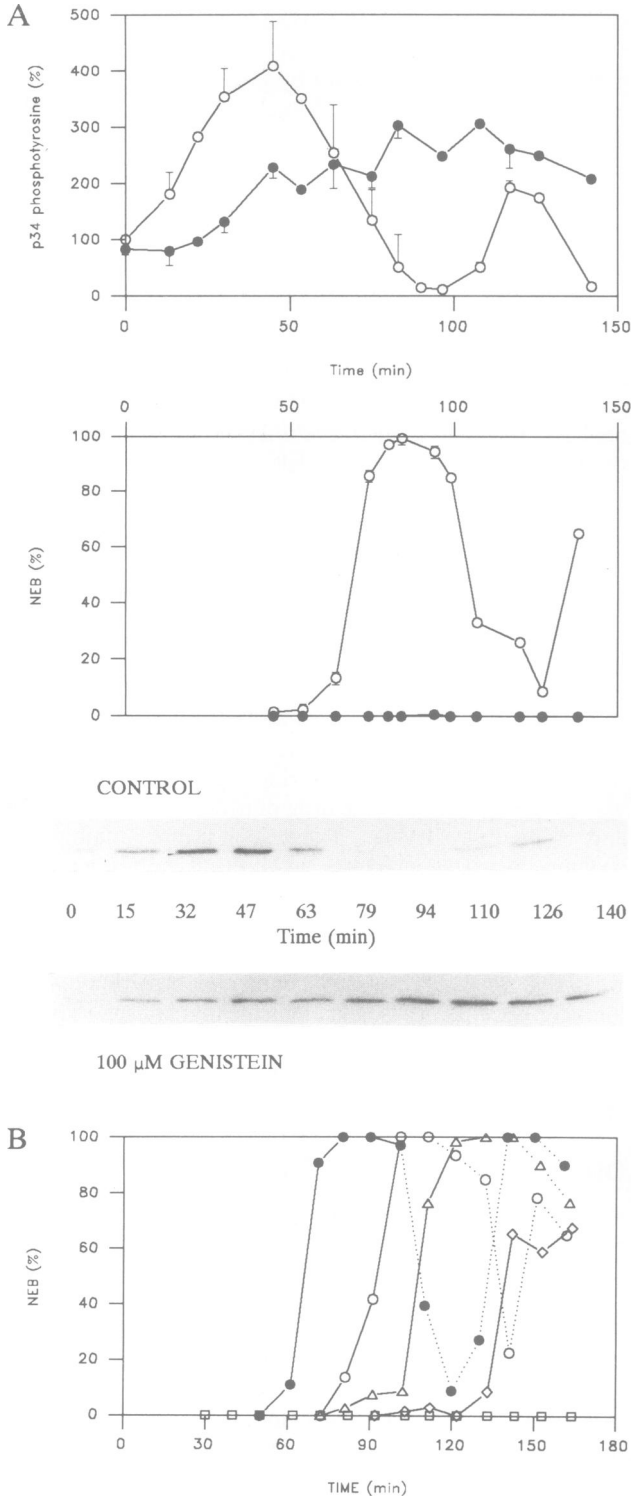

Fig. 4. Genistein blocks NEB and depresses p34 phosphotyrosine accumulation after fertilization. **A**, Top: Quantification: the results of three paired experiments are shown. Peak phosphotyrosine levels in the control embryos were 489 ± 76% (mean and SEM, n = 3). Open symbols: control; closed symbols: genistein (100 μM). Symbols without error bars represent the mean of two of the three experiments. **Bottom:** Autoradiogram of one of the three experiments. **B**, Increasing concentrations of genistein progressively delay mitosis: (●) control; (○) 25 μM genistein; (▲) 50 μM. (◇) 75 μM. *L.pictus*. 16°C.

The p34^{cdc2} tyrosine phosphorylation cycle

The gradual increase in p34 phosphotyrosine after fertilization is followed by loss of phosphotyrosine as the embryo enters mitosis. The temporal correlation of phosphotyrosine loss and entry into mitosis (NEB) is consistent with data from frog and starfish oocytes, frog embryos and cell free extracts, HeLa cells and yeast that indicate that tyrosine dephosphorylation of p34 represents the proximal activation step of the p34 mitotic kinase activity responsible for driving the nucleus into mitosis (Draetta *et al.*, 1988; Gould and Nurse, 1989; Morla *et al.*, 1989; Pondaven *et al.*, 1990; Solomon *et al.*, 1990). We find that during first mitosis in sea urchin embryos, phosphotyrosine levels fall below those measured in unfertilized eggs and tyrosine re-accumulation in interphase of the second cell cycle fails to match the levels achieved during the first, though the relative increase is similar in both cell cycles. Part of the explanation for this discrepancy may lie in the loss of cell cycle synchrony between embryos as development progresses, but the lower levels of p34 phosphotyrosine during first mitosis compared to the unfertilized egg seem genuine and are not an artefact of the p13-bead isolation method (see Materials and methods). We do not know what proportion of p34 is tyrosine phosphorylated. It may be that a greater proportion of p34 is available as a substrate for the tyrosine kinase during the first cell cycle, perhaps as a consequence of the extended interphase cell cycle arrest of the unfertilized egg.

Our experiments with genistein, a tyrosine kinase inhibitor, show that it suppresses p34 tyrosine phosphorylation and delays, then inhibits mitosis in a dose-dependent manner. At first sight, these results suggest that the rate of accumulation of p34 phosphotyrosine determines the timing of mitosis entry. However, even in embryos in which mitosis is suppressed by genistein, p34 phosphotyrosine accumulation occurred, albeit at a much reduced rate, and reached levels that exceeded those found in control embryos immediately prior to mitosis during the second cell cycle. It may be that inhibition of mitosis entry by genistein is an effect unconnected with its inhibition of tyrosine kinase. In yeast, suppressing phosphorylation of p34 on tyrosine 15 advances mitosis, a result that tends to support the argument that genistein blocks mitosis by another route than inhibition of p34 tyrosine phosphorylation.

p34 phosphotyrosine and the cyclin abundance cycle

Sea urchin eggs are arrested in the cell cycle prior to S-phase with very low levels of protein synthesis, and would be expected to possess very low levels of cyclin (Whitaker and Patel, 1990). The cyclins are undetectable by immunoblot in unfertilized sea urchin eggs of the species *Sphaerechinus granularis* and *Strongylocentrotus purpuratus* (Meijer *et al.*, 1989; Tim Hunt, personal communication). We verified that

cyclin in unfertilized *Lytechinus pictus* eggs was also undetectable by immunoblot (see Materials and methods). In addition, we verified that the protein synthesis inhibitor emetine decreased [^{35}S]methionine incorporation into cyclin to $<0.5\%$ of controls (see Materials and methods), effectively suppressing the cyclin abundance cycle.

The p34 phosphotyrosine phosphorylation cycle is similar to its timing to the cyclin abundance cycle (Evans *et al.*, 1983) during the first two embryonic cell cycles. The loss of phosphotyrosine from p34 at mitosis onset precedes the destruction of cyclins that occurs during mitosis, which fits with recent observations in *Xenopus* egg extracts that the active p34^{cdc2}-cyclin complex itself initiates cyclin destruction (Felix *et al.*, 1990). The increase in p34 phosphotyrosine during interphase parallels the interphase

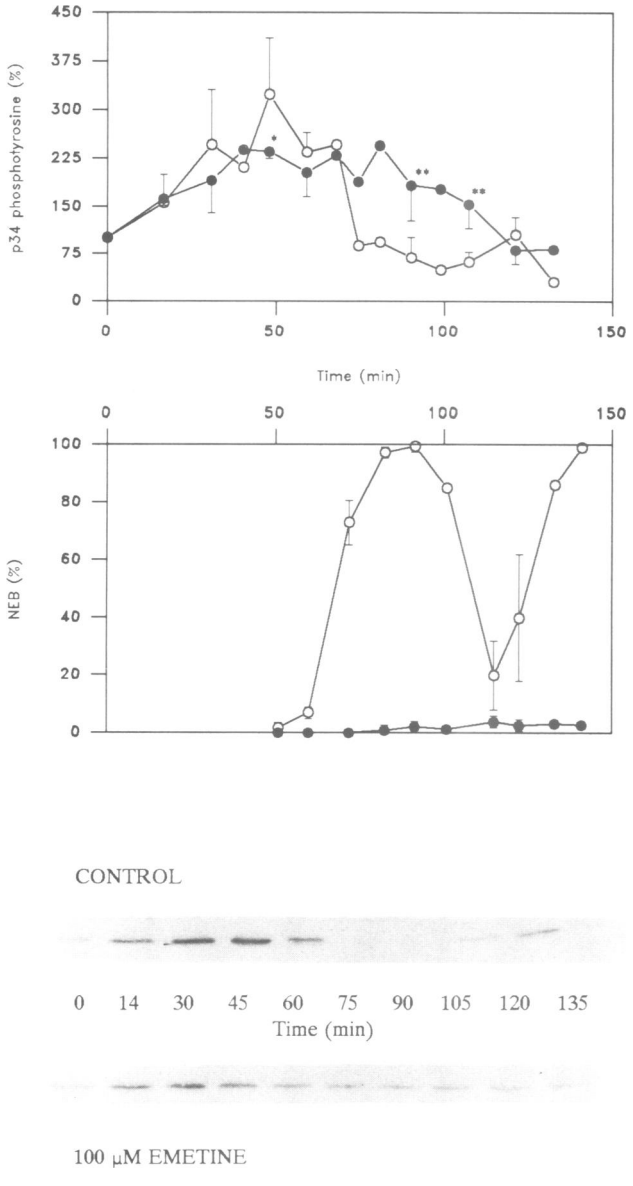

Fig. 5. Emetine, a protein synthesis inhibitor, alters the pattern of p34 tyrosine phosphorylation after fertilization. **Top**: Quantification of p34 phosphotyrosine levels using the anti-phosphotyrosine antibody in three experiments. (○) control. (●) 100 μM emetine. Points with error bars show the mean and SEM. Points without error bars show the mean of two determinations. Control phosphotyrosine levels were 323 ± 87% (mean and SEM) and emetine, 235 ± 10% at 50 min. Phosphotyrosine levels in emetine treated eggs are significantly lower ($P < 0.05$, Student's one-tailed *t*-test:*) and at 90 and 105 min, significantly greater ($P < 0.01$:**). **Bottom:** Autoradiogram of one of the three experiments and its control. *L.pictus* 16°C.

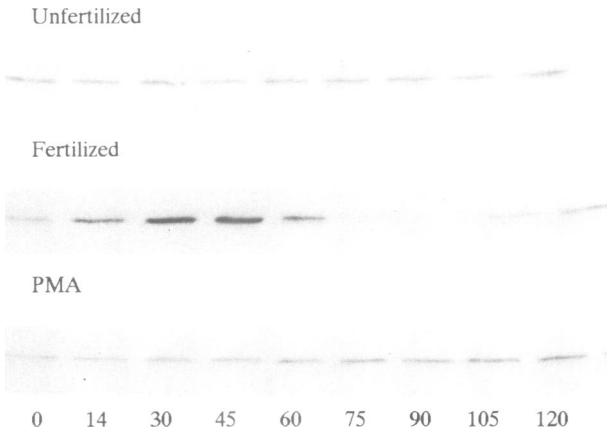

Fig. 6. p34 tyrosine phosphorylation and the fertilization pH_i signal. Unfertilized eggs were treated with phorbol myristate acetate (PMA: 250 nM) in ASW to increase pH_i without increasing Ca_i. The autoradiograms of a representative experiment with the anti-phosphotyrosine antibody are shown, with the corresponding fertilization control. *L.pictus*. 16°C.

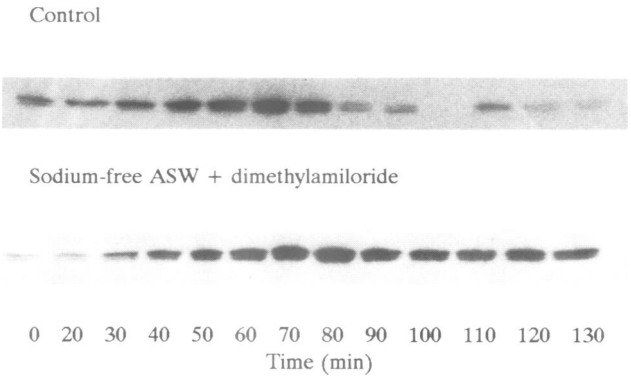

Fig. 7. p34 tyrosine phosphorylation and the fertilization Ca_i signal. The pH_i signal was suppressed by fertilizing eggs in sodium-free ASW containing 100 μM dimethylamiloride. A single experiment is shown, with its control. In sodium-free ASW, sodium chloride is replaced by choline chloride and sodium bicarbonate by potassium bicarbonate. *L.pictus*. 16°C.

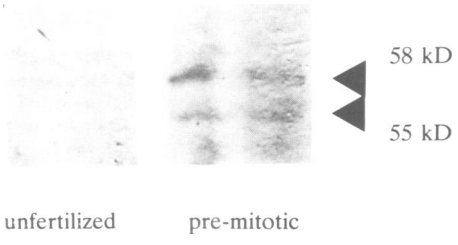

Fig. 8. Cyclin B immunoblot of extracts from unfertilized sea urchin eggs and pre-mitotic (G_2) embryos, 50 min after fertilization. We identify the two bands that co-migrate with the two ^{35}[S]methionine-labelled cyclin bands and disappear during mitosis (not shown) as cyclin and P cyclin, its phosphorylated form. They are visible in pre-mitotic extracts, but undetectable in unfertilized eggs. Undiluted pre-immune serum did not cross-react. *L.pictus*.

increase in cyclins. The close temporal correlation of the two cycles in sea urchin embryos (Meijer *et al.*, 1991) does not appear to reflect a tight coupling, however. We find that preventing cyclin synthesis using a protein synthesis inhibitor or by preventing the fertilization pH_i increase did not prevent the accumulation of phosphotyrosine on p34, nor its subsequent decrease. The p34 phosphotyrosine cycle therefore resembles DNA synthesis (Wagenaar and Mazia, 1978) and the centrosome duplication cycle (Sluder *et al.*, 1990) in being independent of protein synthesis. Nonetheless, like the centrosome duplication cycle, the p34 tyrosine phosphorylation cycle slows when protein synthesis is inhibited.

While our results do not support a tight coupling of cyclin abundance and p34 tyrosine phosphorylation, they do provide evidence of mutual entrainment. The entraining of the accumulation phase of the p34 cycle by protein (cyclin) synthesis is weak, but it does appear from our results that p34 phosphotyrosine is significantly higher ($P < 0.05$) immediately prior to mitosis in controls, compared to emetine-treated embyros. The entrainment may represent the interaction described by Meijer *et al.* (1991) in sea urchin embryos and by Solomon *et al.* (1990), who were able to demonstrate stimulation of p34 tyrosine phosphorylation in *Xenopus* egg extracts by adding a cyclin fusion protein together with vanadate, a presumed tyrosine phosphatase inhibitor. The interaction between the tyrosine dephosphorylation phase of the p34 cycle and protein synthesis is strong, though it is not strictly an entrainment; this linkage can be explained by a mechanism in which auto-activation of the mitotic kinase by tyrosine dephosphorylation is dependent on the kinase's cyclin subunit (Murray, 1989), the mechanism that is also the presently accepted explanation for the inhibition of mitosis onset by inhibitors of protein synthesis (Arion and Meijer, 1989. Solomon *et al.*, 1990).

The tyrosine dephosphorylation phase of the p34 phosphotyrosine cycle in sea urchin embryos persists after inhibition of protein synthesis. The time-course under these conditions presumably reflects the underlying rate-limiting dephosphorylation that determines the timing of mitosis in normal embryos and that is revealed by the absence of auto-activation of the mitotic kinase due to the lack of normal levels of cyclin. The proximal activator of net p34 tyrosine dephosphorylation is not identified in sea urchin embryos, though the distal cause may be completion of DNA synthesis (Sluder and Lewis, 1987).

Materials and methods

Handling of gametes

Eggs of *Lytechinus pictus* (Marinus, CA, USA) were obtained by intracoelomic injection of 0.5 M potassium chloride. The egg jelly was removed by passage of the eggs through Nitex mesh and two washes in artificial sea water (ASW) (450 mM NaCl, 50 mM $MgCl_2$, 11 mM $CaCl_2$, 10 mM KCl, 2.5 mM $NaHCO_3$, 1 mM EDTA, pH 8.0). The dejellied eggs were resuspended as a 10% (v/v) suspension. Sperm was collected 'dry' and stored at 4°C. Eggs were either fertilized or activated parthenogenetically. For fertilization 100 μl of 'dry' sperm was diluted in 100 ml of ASW and added to 100 ml of a 1% (v/v) egg suspension. Fertilization was scored 2–3 min after sperm addition and only batches of eggs showing >95% fertilization were used. The eggs were washed in ASW to remove excess sperm and resuspended as a 10% (v/v) suspension. For parthenogenetic activation the eggs were treated with 200 ml of ASW containing 15 mM NH_4Cl (pH 9.0) for 15 min. After 15 min the eggs were washed twice in ASW and resuspended as a 10% (v/v) suspension. Eggs were kept at 16°C.

Treatment with inhibitors

To assess the effects of the protein synthesis inhibitor emetine on $p34^{cdc2}$ tyrosine phosphorylation, eggs were fertilized in ASW containing 100 μM emetine (Sigma Chemical Co. Ltd, Poole, Dorset, UK). After fertilization the eggs were washed with ASW containing emetine and resuspended as a 10% (v/v) suspension in ASW containing the inhibitor. Parallel experiments were made on each batch of eggs using [^{35}S]methionine and polyacrylamide gel electrophoresis to monitor methionine incorporation into protein (Evans *et al.*, 1983). No discernible bands were present at the 60 min time point (where cyclin is most abundant) after exposure times 20 times longer than required to detect ^{35}S-labelled bands in controls at 30 min (themselves 10% of the radioactivity present at 60 min), implying that the rate of cyclin synthesis was <0.5% that of controls.

To assess the effects of genistein (ICN Biomedicals, High Wycombe, Bucks, UK) on $p34^{cdc2}$ tyrosine phosphorylation eggs were fertilized in ASW containing 100 μM genistein. A stock solution of genistein [20 mM in dimethylsulphoxide (DMSO)] was diluted to 100 μM in ASW. Control eggs were fertilized in ASW containing 0.5% DMSO. After fertilization the eggs were washed in ASW containing genistein and resuspended as a 10% (v/v) suspension in genistein-containing ASW.

Absence of cyclin in unfertilized eggs

Sea urchin eggs are arrested in G_1, prior to S phase and would be expected to contain very low levels of cyclin B (Whitaker and Patel, 1990). We verified this by immunoblotting (Figure 8) with an antibody (serum, 1:10 dilution) raised against a cyclin B (*Arbacia punctulata*)–protein A fusion protein. The fusion protein was provided by Dr Tim Hunt.

p13-Sepharose beads

p13 was purified from a strain of *E.coli* overproducing $p13^{suc1}$ as described previously (Brizuela *et al.*, 1987). Coupling of the p13 to the CnBr-activated Sepharose 4B (Sigma), storage and use of the p13 beads was as described by Meijer *et al.* (1989). The concentration of p13 on the beads was 5 mg/ml-beads. We determined that the amount of p34 bound to the p13 beads was independent of cell cycle stage during the first two embryonic cell cycles by immunoblotting with the PSTAIR antibody.

Preparation of egg extracts

1 ml aliquots of the egg suspension were taken at various times after fertilization or parthenogenetic activation and the eggs rapidly pelleted (10 s) using a hand centrifuge. The supernatant was removed and 400 μl of homogenization buffer (60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM MOPS pH 7.2, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium orthovanadate, 0.1 mM sodium fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 100 μM benzamidine) added to the egg pellet. The extract was immediately frozen in liquid nitrogen and stored at −20°C until required. Before use the extracts were thawed and homogenized by sonication for 10 s and centrifuged at 13 000 *g* for 10 min at 4°C. The supernatant (390 μl) was then added to 400 μl of a 2.5% (v/v) suspension of p13 beads in bead buffer (50 mM Tris–HCl pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 100 μM benzamidine) and the tubes rotated for 60 min at 4°C. The beads were recovered by centrifugation (13 000 *g* for 20 s at 4°C), washed three times with 1 ml of bead buffer, resuspended in 60 μl SDS sample buffer and boiled at 100°C for 3 min.

SDS-PAGE and Western blotting

Proteins bound to the p13 beads were resolved using 10% polyacrylamide gels. For Western blotting the protocol described by Kamps and Sefton (1988) was used, with the exception of the blot of Figure 8, where the protocols described by Sambrook *et al.* (1989) and an alkaline phosphatase-linked goat anti-rabbit secondary antibody (Sigma) were used. Briefly, proteins from the gels were transferred to Immobilon membranes (Millipore) using a transblot cell (Bio-Rad, 60 V for 75 min). The membranes were blocked with blocking buffer containing 5% BSA (essentially fatty acid and immunoglobulin free; Sigma) and 1% ovalbumin (Sigma) in TNA (10 mM Tris-HCl pH 7.4, 0.9% NaCl, 0.01% NaN_3) for 15–30 min at room temperature. The membranes were than incubated overnight at room temperature with an affinity-purified rabbit anti-phosphotyrosine antibody (Kamps and Sefton, 1988) or a mouse anti-phosphotyrosine monoclonal antibody (Glenney *et al.*, 1988) at 2 μg/ml in blocking buffer, washed twice for 10 min in TNA and once for 10 min in TNA supplemented with 0.5% NP-40 and finally twice more in TNA for 5 min. The rabbit anti-phosphotyrosine antibody was detected by incubating the membrane with 2 μCi of [^{125}I]protein A (Amersham, UK) and the mouse with 10 μCi ^{125}I-labelled sheep anti-mouse IgG (Amersham) in 5 ml of blocking buffer for

2 h at room temperature. The membrane was subsequently washed as described above. The membranes were air dried and exposed for autoradiography using Hyperfilm-M (Amersham).

Identification of p34^{cdc2}

p34^{cdc2} was identified on immunoblots using the affinity-purified rabbit anti-PSTAIR p34^{cdc2} antibody at a dilution of 1:500 (Lee and Nurse, 1987). The antibody recognized two closely-migrating bands at 34 and 33 kDa. The anti-phosphotyrosine antibodies recognized both of these bands in immunoblots of unfertilized eggs. We were unable to detect a band corresponding to Eg1, a 32 kDa protein that cross-reacts with the PSTAIR antibody and interacts weakly with p13 beads (Paris *et al.*, 1991; Solomon *et al.*, 1990).

Sources of reagents

All reagents were of Analar grade and from BDH Ltd (Poole, Dorset, UK), unless otherwise indicated.

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