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Cell Cycle Tyrosine Phosphorylation of p34^{cdc2} and a Microtubule-Associated Protein Kinase Homolog in *Xenopus* Oocytes and Eggs

JAMES E. FERRELL, JR.,[1,2]* MICHAEL WU,[3] JOHN C. GERHART,[3] AND G. STEVEN MARTIN[2]

Department of Zoology, University of Wisconsin–Madison, Zoology Research Building, 1117 West Johnson Street, Madison, Wisconsin 53706,[1] and Divisions of Biochemistry and Molecular Biology[2] and Cell and Developmental Biology,[3] University of California, Berkeley, California 94720

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We have examined the time course of protein tyrosine phosphorylation in the meiotic cell cycles of *Xenopus laevis* oocytes and the mitotic cell cycles of *Xenopus* eggs. We have identified two proteins that undergo marked changes in tyrosine phosphorylation during these processes: a 42-kDa protein related to mitogen-activated protein kinase or microtubule-associated protein-2 kinase (MAP kinase) and a 34-kDa protein identical or related to p34^{cdc2}. p42 undergoes an abrupt increase in its tyrosine phosphorylation at the onset of meiosis 1 and remains tyrosine phosphorylated until 30 min after fertilization, at which point it is dephosphorylated. p42 also becomes tyrosine phosphorylated after microinjection of oocytes with partially purified M-phase-promoting factor, even in the presence of cycloheximide. These findings suggest that MAP kinase, previously implicated in the early responses of somatic cells to mitogens, is also activated at the onset of meiotic M phase and that MAP kinase can become tyrosine phosphorylated downstream from M-phase-promoting factor activation. We have also found that p34 goes through a cycle of tyrosine phosphorylation and dephosphorylation prior to meiosis 1 and mitosis 1 but is not detectable as a phosphotyrosyl protein during the 2nd through 12th mitotic cell cycles. It may be that the delay between assembly and activation of the cyclin-p34^{cdc2} complex that p34^{cdc2} tyrosine phosphorylation provides is not needed in cell cycles that lack G2 phases. Finally, an unidentified protein or group of proteins migrating at 100 to 116 kDa increase in tyrosine phosphorylation throughout maturation, are dephosphorylated or degraded within 10 min of fertilization, and appear to cycle between low-molecular-weight forms and high-molecular-weight forms during early embryogenesis.

When fibroblasts are stimulated with mitogens, a number of proteins become phosphorylated at tyrosine residues. Much of the tyrosine phosphorylation is rapid and transient, accompanying the transition from the G0 to G1 phase of the cell cycle (35). Presumably these G0-G1 phosphotyrosyl proteins either carry out the changes in cell organization and biochemistry necessary to prepare for DNA synthesis and cell division or control the activities of other proteins that ultimately carry out these preparations. Several G0-G1 tyrosine-phosphorylated proteins have now been identified, including phospholipase C-γ1 (30, 31, 47), the *ras* GTPase activator protein (GAP) (12, 32), and a 42-kDa serine/threonine-specific protein kinase or group of related kinases variously referred to as p42 or pp42 (2, 6–8, 20, 25), MAP kinase (denoting microtubule-associated protein-2 kinase [37, 38] or mitogen-activated protein kinase [39]), PK42 (16), and ERK1 (4).

Recently, tyrosine phosphorylation has been implicated in the regulation of the G2-M transition as well. Entry into M phase is brought about by the activation of M-phase-promoting factor (MPF), which is a complex of the serine/threonine-specific protein kinase p34^{cdc2} and cyclin B (see reference 27 for a recent review). The *cdc2* protein becomes phosphorylated at tyrosine during G2 phase. The site of this tyrosine phosphorylation has been identified as tyrosine 15 in *Schizosaccharomyces pombe* p34^{cdc2} (21). This residue is situated within the putative ATP-binding site, and its phosphorylation apparently prevents ATP binding. Activation of the kinase activity of p34^{cdc2} and activation of the biological activity of MPF then depend upon dephosphorylation of tyrosine 15 just prior to M phase (11, 22, 34). One putative substrate of p34^{cdc2} is pp60$^{c\text{-}src}$ (33, 41), whose tyrosine kinase activity has been found to increase during M phase (5). This finding raises the possibility that tyrosine phosphorylation not only regulates entry into M phase but regulates events that occur during M phase as well.

We set out to examine cell cycle-associated changes in protein tyrosine phosphorylation in close detail. The cells we have chosen for this study are *Xenopus laevis* oocytes and eggs. These cells are easily obtained in large quantities, arrest spontaneously at specific stages of meiosis (the G2 phase prior to meiosis 1 for oocytes and metaphase of meiosis 2 for unfertilized eggs), and can be induced to undergo meiosis and mitosis in vitro in excellent synchrony. Furthermore, the size of these cells allows macromolecules of interest, such as MPF, to be introduced by microinjection.

Other workers have previously identified two phosphotyrosyl proteins in *Xenopus* oocytes and eggs. The first is a 34-kDa protein related or identical to p34^{cdc2} (11, 42). This protein is tyrosine phosphorylated in G2-arrested oocytes and in activated eggs, but is not tyrosine phosphorylated in unfertilized eggs (11, 17). The precise timing of the phosphorylation and dephosphorylation of p34^{cdc2} has not yet been examined. The second phosphotyrosyl protein is a 42-kDa cytoplasmic protein that may be related to p42/MAP kinase (6, 28).

In this report, we examine tyrosine phosphorylation in synchronized populations of *Xenopus* oocytes and eggs by antiphosphotyrosine immunoblotting, with the particular aim of determining the time course of p34^{cdc2} and p42 tyrosine phosphorylation. We show that p34^{cdc2}, p42, and an unidentified protein or group of proteins of 110 to 116 kDa undergo marked changes in their tyrosine phosphorylation at

* Corresponding author.

the G2-M transition of meiosis 1 and shortly after fertilization; that the changes in tyrosine phosphorylation associated with meiosis 1 can occur downstream from the activation of MPF; and that p100 undergoes cyclical changes in its electrophoretic mobility during the rapid mitotic cell cycles of early embryos. A final unanticipated finding is that tyrosine phosphorylation is not detectable in either $p34^{cdc2}$ or p42 during the 2nd through 12th mitotic cell cycles.

MATERIALS AND METHODS

Isolation of *Xenopus* oocytes, eggs, and sperm. Ovarian tissue was excised from female frogs under benzocaine anesthesia. Frogs were generally not primed with gonadotropin prior to partial ovariectomy. Large immature oocytes were manually dissected from the ovarian tissue and placed in modified Ringer's solution (100 mM NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM $NaHCO_3$). Maturation was induced by addition of progesterone (Sigma; 2 μg/ml, added as a 10,000× concentrate in ethanol) or by microinjection of partially purified MPF (a 30% ammonium sulfate precipitate of clarified egg lysate, roughly three- to fivefold enriched in MPF [49]). Samples of progesterone- and MPF-treated oocytes were collected at various times. The appearance of a white dot at the animal pole was taken to indicate that an oocyte had undergone germinal vesicle breakdown.

In some experiments cycloheximide (Sigma; 20 to 200 μg/ml) was added as an inhibitor of protein synthesis prior to addition of progesterone or microinjection of MPF. The extent of inhibition of protein synthesis was assessed by measuring trichloroacetic acid-precipitable radioactivity in lysates from parallel dishes of [^{35}S]methionine-labeled oocytes (10 μCi/ml, 1 ml/10 oocytes; specific activity, 1,490 Ci/mmol; Amersham).

Male frogs were killed, and their testes were removed and frozen for use in fertilization experiments. Female frogs were induced to lay eggs by dorsal lymph sac injection of human chorionic gonadotropin (Sigma; 800 U). Eggs were collected, placed in modified Ringer's solution diluted with 3 volumes of water, and fertilized by incubation with minced testis. Fertilized eggs were dejellied in 2.5% cysteine-HCl (pH 8.0), washed in 100 mM NaCl, and placed in 1:3-diluted Ringer's solution in glass dishes. Samples of the fertilized eggs were collected at various times after fertilization. Eggs that appeared not to be fertilized and embryos that did not cleave in synchrony with the bulk of the embryos were discarded. Dejellied, washed eggs were parthenogenetically activated by a 12-V electric shock for 1 s.

Lysis. Ten oocytes, eggs, or embryos were added to 100 μl of ice-cold 40 mM NaCl–10 mM Tris–10 mM EDTA–2 mM phenylmethylsulfonyl fluoride (PMSF)–1 mM Na_3VO_4 (pH 7.5) and lysed by mechanical agitation through a 200-μl pipette tip. Lysates were centrifuged for 90 s in a microcentrifuge with a right-angle rotor. Samples of clarified cytoplasm (80 μl) were removed from the overlying lipid and underlying yolk protein, added to 20 μl of 5× Laemmli gel sample buffer (26), and frozen in a dry-ice bath. For immunoprecipitation experiments, samples of clarified cytoplasm were dissolved in 5× RIPA buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.0], 10 mM EDTA [pH 8.0], 5% Nonidet P-40, 5% sodium deoxycholate, 5% aprotinin solution, 5 mM sodium orthovanadate, 1 mM PMSF, 500 μg of leupeptin per ml) and frozen as above.

Antibodies. Antiphosphotyrosine antiserum was raised as described before (9, 23) and purified in one of two ways. Affinity-purified antiphosphotyrosine antibody was prepared by chromatography on phosphotyramine-Affigel 10 (9), essentially as described earlier (23). The yield of affinity-purified antibody was less than 1% of the initial immunoglobulin G (IgG). For a few experiments, total IgG from immunized rabbits was used, prepared by chromatography on protein A (Beckman) with a yield of about 80%. Somewhat surprisingly, both the affinity-purified and total IgG preparations appeared to recognize the same *Xenopus* proteins in immunoblots and did so with roughly the same titers. Antiphosphotyrosine antibody was used at a working concentration of 2 μg/ml in Tris-saline containing 5% albumin. Preincubation of either the affinity-purified antibody or total IgG preparations with phenyl phosphate (40 mM) blocked recognition of all *Xenopus* proteins.

An anti-ERK1 antiserum was generously provided by Melanie Cobb and used at a dilution of 1:100 for immunoblotting. An antibody raised against bacterially expressed GAP (*ras*-GTPase activating protein) was generously provided by Christine Ellis and Tony Pawson (12) and used at a dilution of 1:200 for immunoblotting and 1:20 for immunoprecipitation. Anti-PSTAIR antibodies were generously provided by Mark Solomon and Mark Kirschner(42) and used at a dilution of 1:200 for immunoblotting. $p13^{suc1}$ beads were also provided by Solomon and Kirschner and were used as described previously (42).

Electrophoresis and blotting. Lysates were subjected to electrophoresis on 10% polyacrylamide Laemmli gels (23) (acrylamide/bisacrylamide ratio, 37.5). Proteins were transferred to Immobilon P membranes (Millipore) at 5 V/cm for 90 min in a tank blotter at 4°C. Transfer buffer contained 192 mM glycine and 25 mM Tris base. Blots were blocked with 5% bovine serum albumin (ICN; Pentax grade) in Tris-saline (140 mM NaCl, 10 mM Tris [pH 7.4]) for 1 h at room temperature, incubated with antiphosphotyrosine antiserum, anti-ERK1 antiserum, anti-PSTAIR antiserum, or anti-GAP antiserum for 1 h at room temperature, washed (two 10-min washes with Tris-saline, one 10-min wash with Tris-saline plus 0.05% Nonidet P-40, and then two 10-min washes with Tris-saline), incubated with ^{125}I-protein A (ICN; specific activity, approximately 40 μCi/μg), and washed again as detailed above. Autoradiography was carried out with Kodak X-Omat AR film and a Du Pont Cronex intensifying screen.

Immunoprecipitation. GAP immunoprecipitations were carried out by preclearing RIPA lysates (200 μl) with 100 μl of 10% Formalin-fixed *Staphylococcus aureus* (IgSorb; The Enzyme Center), incubating the precleared lysates with antibody (10 μl) or normal rabbit serum for 1 h, collecting the immune complexes on *S. aureus*, and washing the complexes with 1 M NaCl–10 mM Tris (pH 7.4)–0.1% Nonidet P-40, then with 1× RIPA buffer, and finally with 10 mM Tris (pH 7.4)–0.1% Nonidet P-40. Proteins were eluted from the *S. aureus* cells by incubation in 1× Laemmli sample buffer.

RESULTS

Tyrosine phosphorylation during oocyte maturation. Oocytes were treated with progesterone in vitro to induce maturation. Progesterone-treated oocytes were collected at hourly intervals and lysed, and protein-tyrosine phosphorylation was assessed by antiphosphotyrosine immunoblotting. As shown in Fig. 1, dramatic changes took place in the tyrosine phosphorylation of proteins in three regions, 34, 42, and approximately 116 kDa.

Tyrosine-phosphorylated p34 was detectable in immature oocytes and persisted until 1 h before germinal vesicle

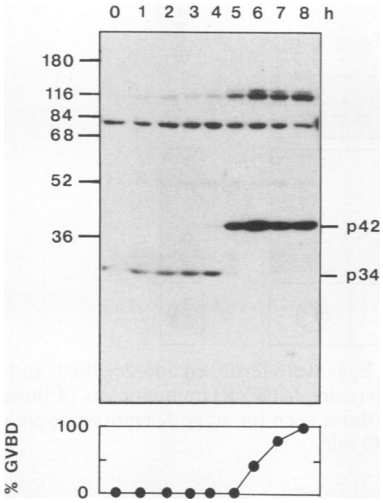

FIG. 1. Time course of tyrosine phosphorylation during progesterone-induced *Xenopus* oocyte maturation. A population of oocytes was treated with progesterone, and samples of 10 cells were taken at hourly intervals. The top panel shows an antiphosphotyrosine immunoblot of those samples. Sizes (in kilodaltons) are shown at the left. The bottom panel shows the percentage of oocytes in each sample that had undergone GVBD. At each time point, the sample percent GVBD was within 10% of the population percent GVBD.

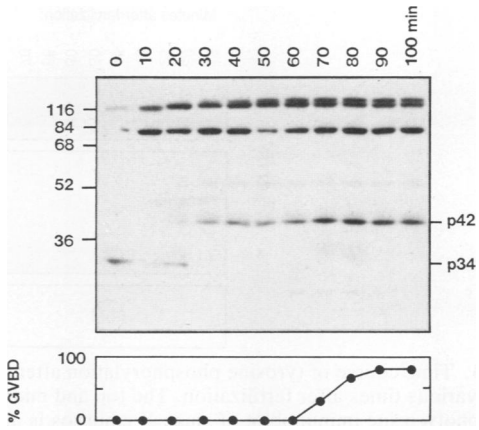

FIG. 2. Tyrosine phosphorylation in cycloheximide-treated *Xenopus* oocytes after microinjection of partially purified MPF. Oocytes were incubated with 20 μg of cycloheximide per ml for 15 min and then microinjected with partially purified MPF. Samples were taken at various times after MPF microinjection and processed for antiphosphotyrosine immunoblotting (top panel). Sizes (in kilodaltons) are shown at the left. The bottom panel shows the percent GVBD in each sample.

breakdown, at which point the tyrosine-phosphorylated p34 abruptly disappeared (Fig. 1). The decrease in p34 tyrosine phosphorylation coincided with an equally abrupt increase in the tyrosine phosphorylation of p42. The concurrence of the changes in p42 and p34 tyrosine phosphorylation raises the possibility of a causal link between the two.

A group of closely spaced bands migrating at 110 to 120 kDa, here collectively called p116, was detectable within 1 h of progesterone treatment. p116 underwent a small increase in tyrosine phosphorylation over the next few hours and then increased dramatically in tyrosine phosphorylation at about the time of germinal vesicle breakdown (Fig. 1). p116 also progressively increased in its apparent molecular mass. These findings suggest that p116 represents one protein changing in electrophoretic mobility due to increasing phosphorylation stoichiometry. Alternatively, p116 could be a collection of different proteins being phosphorylated in succession.

No further changes in protein tyrosine phosphorylation were detected after germinal vesicle breakdown (Fig. 1 and data not shown). The pattern of tyrosine phosphorylation seen after germinal vesicle breakdown was indistinguishable from that seen in unfertilized eggs (Fig. 1 and 5 and data not shown).

Protein-tyrosine phosphorylation following MPF microinjection. To assess whether protein synthesis was required for the appearance of phosphotyrosyl p42 and p116 and to assess the relationship between MPF activation and the tyrosine phosphorylation of these proteins, we treated oocytes with cycloheximide to inhibit protein synthesis and microinjected partially purified MPF to initiate oocyte maturation. The concentration of cycloheximide used (20 μg/ml) was found to maximally inhibit protein synthesis and progesterone-stimulated maturation (not shown) but does not inhibit MPF-stimulated MPF activation and germinal vesicle breakdown (19) (Fig. 2).

Microinjection of cycloheximide-treated oocytes with MPF caused the endogenous phosphotyrosyl p34 to disappear and caused phosphotyrosyl p42 to appear (Fig. 2). These phosphorylation changes were similar to those seen in non-cycloheximide-treated oocytes after progesterone treatment except that p42 phosphorylation and p34 dephosphorylation took place about 30 min after MPF microinjection (Fig. 2) and about 5 h after progesterone treatment (Fig. 1). p116 also increased in tyrosine phosphorylation after MPF microinjection of cycloheximide-treated oocytes. Similar changes were seen in p34, p42, and p116 tyrosine phosphorylation in non-cycloheximide-treated oocytes after MPF microinjection (not shown). The most straightforward interpretation of these findings is that the appearance of phosphotyrosyl p42 and p116 represents an increase in the tyrosine phosphorylation of p42 and p116 molecules that are already present in non-tyrosine-phosphorylated form in G2-arrested oocytes and that the tyrosine phosphorylation of p42 and p116 and dephosphorylation of p34 can occur downstream from MPF activation.

Protein-tyrosine phosphorylation in fertilized eggs. We examined changes in egg protein-tyrosine phosphorylation following two types of activation. The first was fertilization, which allows the synchrony of the cleaving eggs to be monitored. Eggs were fertilized and dejellied, and tyrosine phosphorylation was assessed during the completion of meiosis 2 and the subsequent mitotic cycles. Phosphotyrosyl p42 was found to disappear about 30 min after fertilization (Fig. 3, middle panel). The disappearance of p42 coincided with the reappearance of phosphotyrosyl p34. Phosphotyrosyl p34 was present until just prior to the first egg cleavage (Fig. 3, middle panel). No tyrosine phosphorylation of either p42 or p34 was detected during the second or third mitotic cell cycles (Fig. 3; two similar experiments not shown). These cycles differ from the first mitotic cell cycle in that they are more rapid and lack G2 phases. Phosphotyrosyl p34 but not phosphotyrosyl p42 reappeared in stage VIII and later embryos (Fig. 3 and data not shown). Stage VIII corresponds to the midblastula transition, the time at which

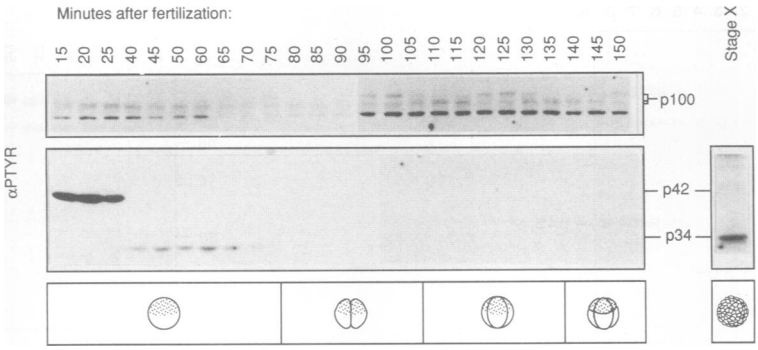

FIG. 3. Time course of tyrosine phosphorylation after fertilization of *Xenopus* eggs. Eggs were fertilized and dejellied, and samples were taken at various times after fertilization. The top and middle panels show antiphosphotyrosine (αPTYR) immunoblots of those samples. An antiphosphotyrosine immunoblot of stage X embryos is also shown. Results similar to those seen for stage X embryos were obtained from stage VIII and IX embryos as well (not shown). Cleavages occurred at 80, 110, and 140 min.

mitosis becomes desynchronized and slows down and G2 phases reappear.

The top panel of Fig. 3 shows antiphosphotyrosine blots of the same samples carried out at a higher transfer voltage to improve the transfer of the proteins of 100 to 116 kDa (at the expense of p34 and other low-molecular-mass proteins). A group of three closely spaced phosphotyrosyl protein bands migrating between 100 and 116 kDa underwent successive cyclical changes in their prominence during the mitotic cell cycles. This oscillation suggests that the serine or threonine phosphorylation of a 100-kDa phosphotyrosyl protein might undergo cyclical changes. Alternatively, the bands could represent unrelated proteins appearing and disappearing in succession. The relationship between the 116-kDa protein that becomes tyrosine phosphorylated during maturation and the cycling 100- to 116-kDa protein(s) is uncertain; we will refer to the latter collectively as p100 and continue to call the former p116. The higher-molecular-mass forms of p100 peaked about midway between cell cleavages.

Identification of tyrosine-phosphorylated proteins. The 34-kDa phosphotyrosyl protein described here was found to bind to $p13^{suc1}$ beads and comigrate with a protein recognized by anti-PSTAIR antibodies (Fig. 4 and data not shown). These findings confirm previous reports (11, 17, 42) and suggest that p34 is related or identical to the *cdc2* protein. Other workers have detected a 34-kDa phosphotyrosyl protein in *Xenopus* egg extracts and have demonstrated that it is distinct from the Eg1 protein (42), a protein related to $p34^{cdc2}$ with similar $p13^{suc1}$ and anti-PSTAIR binding properties (36). We have likewise found that our p34 migrates above authentic Eg1 protein (not shown). It therefore appears likely that the 34-kDa phosphotyrosyl protein seen here and elsewhere (11, 17, 42) is $p34^{cdc2}$ or some as yet unidentified *cdc2*-related protein.

A variety of indirect approaches have suggested that a 42-kDa *Xenopus* phosphotyrosyl protein is a member of the MAP kinase family of serine/threonine-specific protein kinases (6, 16, 37–39). More recently, peptide sequences from purified *Xenopus* p42 have been found to closely resemble sequences in the protein kinase domain and ERK-specific C-terminal tail of the ERK1 and ERK2 proteins (4, 15a). These findings provide direct evidence that, as hypothesized, p42 is a MAP kinase or MAP kinase-like protein.

The molecular mass of p116 is similar to that of GAP, and GAP becomes tyrosine phosphorylated when fibroblasts are stimulated with various mitogens (12, 32). However, GAP immunoprecipitated from pre- and post-germinal vesicle breakdown (GVBD) *Xenopus* oocytes was not recognized by antiphosphotyrosine antibodies (not shown). Thus, it appears that p116 is not GAP, and the identity (or identities) of p116 remains uncertain.

p34 and p42 abundance after fertilization. Using antibodies that recognize both the tyrosine-phosphorylated and unphosphorylated forms of $p34^{cdc2}$ and p42, we assessed whether the changes seen in their phosphotyrosine content after fertilization represented changes in phosphorylation stoichiometry or protein abundance.

Anti-ERK1 antibodies recognized purified *Xenopus* p42

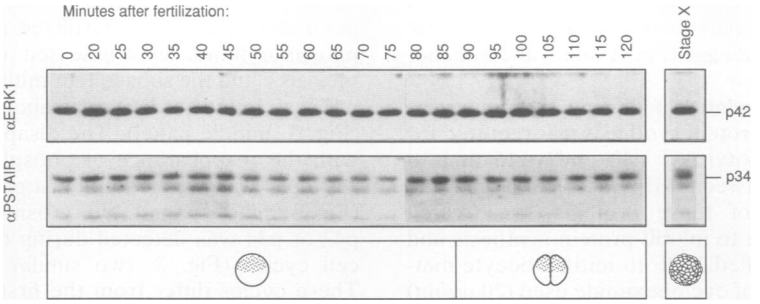

FIG. 4. Abundances of p34 and p42 after fertilization of *Xenopus* eggs. Eggs were fertilized and dejellied, and samples were taken at various times. Unfertilized eggs and stage X embryos are also shown. The upper panel shows anti-ERK1 (αERK1) immunoblots. Only the region between 40 and 50 kDa is shown. The middle panel shows anti-PSTAIR (αPSTAIR) immunoblots. The region between about 25 and 40 kDa is shown. Cleavages occurred at 90 and 120 min.

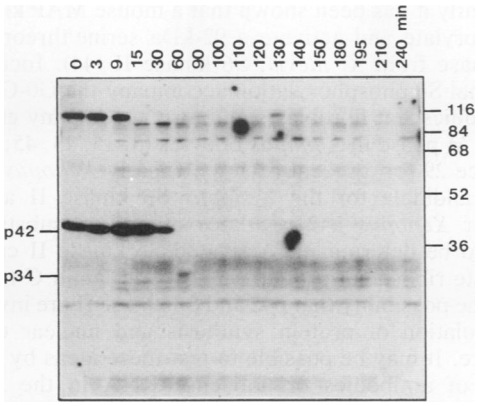

FIG. 5. Time course of tyrosine phosphorylation after parthenogenetic activation of *Xenopus* eggs. Eggs were dejellied and activated, and samples were taken at various times and processed for antiphosphotyrosine immunoblotting. The 34-kDa band seen 60 min after fertilization may be p34; however, background bands with similar mobilities render this identification tentative (cf. Fig. 3).

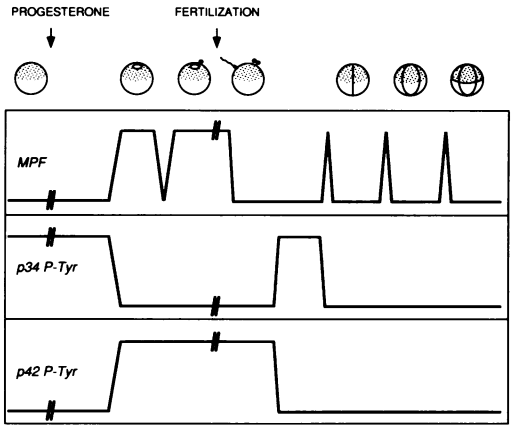

FIG. 6. Schematic view of the changes in MPF activity, p34 tyrosine phosphorylation, and p42 tyrosine phosphorylation during progesterone-induced maturation and after fertilization. Changes in MPF activity are taken from reference 19; p34 and p42 tyrosine phosphorylation data are from the present work. P-Tyr, Phosphotyrosine.

(not shown) and recognized a 42-kDa protein in egg lysates that comigrated and copurified with phosphotyrosyl p42 (Fig. 4 and data not shown). The intensity of the 42-kDa band on anti-ERK1 immunoblots remained constant from fertilization through early embryogenesis to stage X (Fig. 4). The abrupt decrease in phosphotyrosyl p42 seen 30 min after fertilization therefore represents a decrease in phosphorylation stoichiometry rather than protein abundance. This decrease could be brought about by dephosphorylation of p42 or proteolysis of phosphotyrosyl p42 accompanied by synthesis of an equal number of non-tyrosine-phosphorylated p42 molecules.

Anti-PSTAIR antibodies recognized three protein bands in the 30- to 40-kDa range (Fig. 4). The most prominent of the three was a 34-kDa band that comigrated with the 34-kDa phosphotyrosyl protein seen before mitosis 1 and after the midblastula transition (not shown). No consistent changes were seen in the intensities of any of the PSTAIR protein bands after fertilization, during the early mitotic cell cycles, or during early embryogenesis through stage X (Fig. 4). These findings suggest that p34 undergoes a transient increase in its tyrosine phosphorylation stoichiometry during the first mitotic cell cycle without changing in abundance. These findings also suggest that the lack of detectable tyrosine-phosphorylated p34 during the 2nd through 12th mitotic cell cycles is not due to a decrease in the abundance of the protein.

Protein-tyrosine phosphorylation in parthenogenetically activated eggs. To allow examination of tyrosine phosphorylation in the first few minutes after activation, we parthenogenetically activated dejellied eggs by electric shock. The first change detected was a marked decrease in the intensity of the 116-kDa phosphotyrosyl protein (Fig. 5). This disappearance preceded the disappearance of phosphotyrosyl p42 and occurred approximately when MPF disappears after activation (19). The less prominent phosphotyrosyl p100 band became detectable at the same time. Through the subsequent abortive cleavages, p100 appeared to cycle between low-mobility and high-mobility forms.

Phosphotyrosyl p42 disappeared about 30 min after parthenogenetic activation, followed by the transient tyrosine phosphorylation of p34 (Fig. 5), as was seen following fertilization (Fig. 3). Neither p42 nor p34 reappeared as a phosphotyrosyl protein during the first few abortive cleavages (Fig. 5). These findings demonstrate that the changes in tyrosine phosphorylation seen after fertilization do not depend upon sperm components.

DISCUSSION

Proteins with molecular masses on sodium dodecyl sulfate gels of 34, 42, and 100 to 120 kDa undergo marked changes in their phosphotyrosine content during *Xenopus* oocyte maturation and after egg fertilization. The 34-kDa protein comigrates with a protein recognized by anti-PSTAIR antibodies and binds to $p13^{suc1}$ beads, suggesting that it is either the *cdc2* protein or a closely related protein. Sequence data and antibody cross-reactivity show that p42 is related to the ERK1 protein, a rat brain MAP kinase. The identities of the 100- to 120-kDa proteins are uncertain.

Figure 6 summarizes, in highly schematic fashion, how the time courses of p34 tyrosine phosphorylation, p42 tyrosine phosphorylation, and MPF activation relate to each other. p34 undergoes an abrupt decrease in its tyrosine phosphorylation to undetectable levels about 1 h prior to germinal vesicle breakdown. This finding is consistent with the growing body of evidence that dephosphorylation of $p34^{cdc2}$ is necessary for the kinase activity and biological activity of MPF to become manifest (11, 17, 22, 34). The next change in the tyrosine phosphorylation of p34 occurs during the G2 phase that precedes the first mitotic division. Here p34 becomes transiently tyrosine phosphorylated, losing its tyrosine phosphorylation when MPF becomes active prior to mitosis 1.

After completion of mitosis 1, the cell undergoes rapid cell cycles that lack G1 and G2 phases. These cell cycles are accompanied by periodic spikes of MPF activity (19); however, we never detected cycles of p34 tyrosine phosphorylation and dephosphorylation until about the time of the midblastula transition, when phosphotyrosyl p34 becomes detectable again. The apparent lack of $p34^{cdc2}$ tyrosine phosphorylation distinguishes the cleaving embryo from cycling extracts, which exhibit cyclical changes in $p34^{cdc2}$ tyrosine phosphorylation through multiple cycles (9a, 41a).

It may be that tyrosine-phosphorylated p34^{cdc2} does not accumulate in cell cycles that lack G2 phases, either because phosphorylation does not occur or because some phosphotyrosine phosphatase is constitutively active. It is clear that tyrosine phosphorylation of p34^{cdc2} is one way of inhibiting its kinase activity and thereby introducing a temporal gap between the assembly of the cyclin-p34^{cdc2} complex and its activation (22). The present work raises the possibility that the tyrosine phosphorylation step may be dispensed with if such a gap is not needed.

These results could explain the observation that the *Xenopus* p34^{cdc2} cycle does not require successful completion of DNA synthesis until the time of the midblastula transition (24). Enoch and Nurse have shown that in *S. pombe*, overexpression of the *cdc25*$^{+}$ protein, which may be a positive regulator of a phosphotyrosine phosphatase (21), abolishes the dependence of mitosis on DNA synthesis (14). Thus, if the requirement for DNA synthesis is exerted through control of a phosphotyrosine phosphatase, then either constitutive activation of the phosphatase or elimination of the p34^{cdc2} tyrosine phosphorylation step could abrogate this control.

It is not yet known whether threonine phosphorylation sites in p34^{cdc2} undergo cyclical changes in phosphorylation during the 2nd through 12th mitotic cell cycles or whether the protein is locked in some particular phosphorylation state. Likewise, it is not known whether cyclin B undergoes cyclical changes in its phosphorylation during these cell cycles. It will be of interest to determine whether any biochemical changes beyond cyclin synthesis and p34^{cdc2}-cyclin complex formation are required for MPF activation in cycles 2 through 12.

p42 becomes tyrosine phosphorylated during oocyte maturation concomitantly with the dephosphorylation of p34. The tyrosine phosphorylation of p42 can occur in response to MPF activation and can occur in the absence of protein synthesis. Thus, p42 is present in a non-tyrosine-phosphorylated form in oocytes and may be involved downstream from MPF in a cascade that brings about meiotic M phase. By analogy with fibroblast MAP kinase, the serine/threonine kinase activity of *Xenopus* p42 may depend upon tyrosine phosphorylation. It is also possible that other modifications in addition to tyrosine phosphorylation are required in the activation of p42 (1). We do not know whether such modifications accompany oocyte maturation, although we do know that purified p42 from *Xenopus* eggs is active as a protein kinase.

p42 becomes dephosphorylated without decreasing in abundance about 30 min after fertilization. This dephosphorylation coincides with an increase in the tyrosine phosphorylation of p34. At present we do not know whether tyrosine-phosphorylated p42 acts to inhibit one of the steps leading up to p34 tyrosine phosphorylation or whether the temporal correspondence between these events is fortuitous. p42 remains dephosphorylated, and presumably inactive as a protein kinase, through the midblastula transition.

Thus, MAP kinase or MAP kinase-like proteins are tyrosine phosphorylated, and possibly activated, at two different points in two different cell cycles: during the G0-G1 transition in mitogen-stimulated fibroblasts, and during meiotic M phase in oocyte maturation. Our working hypothesis is that MAP kinase regulates cellular processes that take place whenever quiescent cells, be they quiescent fibroblasts or G2-arrested oocytes, reenter the cell cycle. MAP kinase may also play an important role in cells that do not divide (13).

Recently it has been shown that a mouse MAP kinase can phosphorylate and activate a 92-kDa serine/threonine protein kinase from *X. laevis*, S6 kinase II (44). Increases in ribosomal S6 phosphorylation accompany the G0-G1 transition in quiescent fibroblasts and also accompany entry into meiotic M phase in *Xenopus* oocytes (3, 15, 43, 45; see also reference 29 for a recent review). Clearly *Xenopus* p42 is a likely candidate for the *Xenopus* S6 kinase II activator. Whether *Xenopus* p42 regulates other key substrates remains to be determined. Activated S6 kinase II can phosphorylate ribosomal protein S6 (15) and lamin C (48). This raises the possibility that p42 and S6 kinase II are involved in the regulation of protein synthesis and nuclear envelope structure. It may be possible to test these ideas by microinjection of antibodies or antisense RNA in the *Xenopus* system. Alternatively, if homologs of these proteins are present in genetically tractable organisms, a genetic analysis of the function of p42 and S6 kinase II may be feasible.

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REFERENCES

1. **Anderson, N. G., J. L. Maller, N. K. Tonks, and T. W. Sturgill.** 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature (London) **343**:651–653.
2. **Bishop, R., R. Martinez, K. D. Nakamura, and M. J. Weber.** 1983. A tumor promoter stimulates phosphorylation on tyrosine. Biochem. Biophys. Res. Commun. **115**:536–543.
3. **Blenis, J., and R. L. Erikson.** 1985. Regulation of a ribosomal protein S6 kinase activity by the Rous sarcoma virus transforming protein, serum, or phorbol ester. Proc. Natl. Acad. Sci. USA **82**:7621–7625.
4. **Boulton, T. G., G. D. Yancopoulos, J. S. Gregory, C. Slaughter, C. Moomaw, J. Hsu, and M. H. Cobb.** 1990. An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science **249**:64–67.
5. **Chakalaparampil, I., and D. Shalloway.** 1988. Altered phosphorylation and activation of pp60$^{c\text{-}src}$ during fibroblast mitosis. Cell **52**:801–810.
6. **Cooper, J. A.** 1989. Related proteins are phosphorylated at tyrosine in response to mitogenic stimuli and at meiosis. Mol. Cell. Biol. **9**:3143–3147.
7. **Cooper, J. A., and T. Hunter.** 1985. Major substrate for growth factor-activated protein-tyrosine kinases is a low-abundance protein. Mol. Cell. Biol. **5**:3304–3309.
8. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1984. Diverse mitogenic agents induce the phosphorylation of two related 42,000-dalton proteins on tyrosine in quiescent chick cells. Mol. Cell. Biol. **4**:30–37.
9. **Dailey, D., G. L. Schieven, M. Y. Lim, H. Marquardt, T. Gilmore, J. Thorner, and G. S. Martin.** 1990. Novel yeast protein kinase (*YPK1* gene product) is a 40-kilodalton phosphotyrosyl protein associated with protein-tyrosine kinase activity. Mol. Cell. Biol. **10**:6244–6256.

9a. **Dunphy, W.** Personal communication.

10. **Dunphy, W. G., L. Brizuela, D. Beach, and J. Newport.** 1988. The *Xenopus cdc2* protein is a component of MPF, a cytoplas-

mic regulator of mitosis. Cell **54**:423–431.
11. **Dunphy, W. G., and J. W. Newport.** 1989. Fission yeast p13 blocks mitotic activation and tyrosine dephosphorylation of the *Xenopus cdc2* protein kinase. Cell **58**:181–191.
12. **Ellis, C., M. Moran, F. McCormick, and T. Pawson.** 1990. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature (London) **343**: 377–381.
13. **Ely, C. M., K. M. Oddie, J. S. Litz, A. J. Rossomando, S. B. Kanner, T. W. Sturgill, and S. J. Parsons.** 1990. A 42-kD tyrosine kinase substrate linked to chromaffin cell secretion exhibits an associated MAP kinase activity and is highly related to a 42-kD mitogen-stimulated protein in fibroblasts. J. Cell Biol. **110**:731–742.
14. **Enoch, T., and P. Nurse.** 1990. Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. Cell **60**:665–673.
15. **Erikson, E., and J. L. Maller.** 1986. Purification and characterization of a protein kinase from *Xenopus* eggs highly specific for ribosomal protein S6. J. Biol. Chem. **261**:350–355.
15a. **Ferrell, J., A. Admon, M. Wu, J. Gerhart, and G. S. Martin.** Unpublished data.
16. **Ferrell, J. E., and G. S. Martin.** 1990. Identification of a 42-kilodalton phosphotyrosyl protein as a serine(threonine) protein kinase by renaturation. Mol. Cell. Biol. **10**:3020–3026.
17. **Gautier, J., T. Matsukawa, P. Nurse, and J. Maller.** 1989. Dephosphorylation and activation of *Xenopus* p34^{cdc2} protein kinase during the cell cycle. Nature (London) **339**:626–629.
18. **Gautier, J., C. Norbury, M. Lohka, P. Nurse, and J. Maller.** 1988. Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2*$^+$. Cell **54**:433–439.
19. **Gerhart, J., M. Wu, and M. Kirschner.** 1984. Cell cycle dynamics of an M-phase-specific cytoplasmic factor in *Xenopus laevis* oocytes and eggs. J. Cell Biol. **98**:1247–1255.
20. **Gilmore, T., and G. S. Martin.** 1983. Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. Nature (London) **306**:487–490.
21. **Gould, K. L., S. Moreno, N. K. Tonks, and P. Nurse.** 1990. Complementation of the mitotic activator, p80^{cdc25}, by a human protein-tyrosine phosphatase. Science **250**:1573–1576.
22. **Gould, K. L., and P. Nurse.** 1989. Tyrosine phosphorylation of the fission yeast cdc2$^+$ protein kinase regulates entry into mitosis. Nature (London) **342**:39–45.
23. **Kamps, M. P., and B. M. Sefton.** 1988. Identification of multiple novel polypeptide substrates of the v-*src*, v-*yes*, v-*fps*, v-*ros*, and v-*erb*B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. Oncogene **2**:305–315.
24. **Kimmelman, D., M. Kirschner, and T. Scherson.** 1987. The events of the midblastula transition in Xenopus are regulated by changes in the cell cycle. Cell **48**:399–407.
25. **Kohno, M.** 1985. Diverse mitogenic agents induce rapid phosphorylation of a common set of cellular proteins at tyrosine in quiescent mammalian cells. J. Biol. Chem. **260**:1771–1779.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
27. **Lewin, B.** 1990. Driving the cell cycle: M phase kinase, its partners, and substrates. Cell **61**:743–752.
28. **Lohka, M. J., J. L. Kyes, and J. L. Maller.** 1987. Metaphase protein phosphorylation in *Xenopus laevis* eggs. Mol. Cell. Biol. **7**:760–768.
29. **Maller, J. L.** 1990. *Xenopus* oocytes and the biochemistry of cell division. Biochemistry **29**:3157–3166.
30. **Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger.** 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signalling. Cell **57**:1101–1107.
31. **Meisenhelder, J., P.-G. Suh, S. G. Rhee, and T. Hunter.** 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases *in vivo* and *in vitro*. Cell **57**:1109–1122.
32. **Molloy, C. J., D. P. Bottaro, T. P. Fleming, M. S. Marshall, J. B. Gibbs, and S. A. Aaronson.** 1989. PDGF induction of tyrosine phosphorylation of GTPase activating protein. Nature (London) **342**:711–714.
33. **Morgan, D. O., J. M. Kaplan, J. M. Bishop, and H. E. Varmus.** 1989. Mitosis-specific phosphorylation of p60$^{c\text{-}src}$ by p34^{cdc2}-associated protein kinase. Cell **57**:775–786.
34. **Morla, A. O., G. Draetta, D. Beach, and J. Y. J. Wang.** 1989. Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entry into mitosis. Cell **58**:193–203.
35. **Morla, A. O., and J. Y. J. Wang.** 1986. Protein tyrosine phosphorylation in the cell cycle of BALB/c 3T3 fibroblasts. Proc. Natl. Acad. Sci. USA **83**:8191–8195.
36. **Paris, J., R. Le Guellec, A. Couturier, K. Le Guellec, F. Omilli, J. Camonis, S. MacNeill, and M. Philippe.** Proc. Natl. Acad. Sci. USA, in press.
37. **Ray, L. B., and T. W. Sturgill.** 1987. Rapid stimulation of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 *in vitro*. Proc. Natl. Acad. Sci. USA **84**:1502–1506.
38. **Ray, L. B., and T. W. Sturgill.** 1988. Characterization of insulin-stimulated microtubule-associated protein kinase. J. Biol. Chem. **263**:12721–12727.
39. **Rossomando, A. J., M. J. Weber, and T. W. Sturgill.** 1989. Evidence that pp42, a major tyrosine kinase target protein, is MAP kinase, a mitogen-activated serine/threonine protein kinase. Proc. Natl. Acad. Sci. USA **86**:6940–6943.
40. **Sanghera, J. S., H. B. Paddon, S. A. Bader, and S. L. Pelech.** 1990. Purification and characterization of a maturation-activated myelin basic protein kinase from sea star oocytes. J. Biol. Chem. **265**:52–57.
41. **Shenoy, S., J.-K. Choi, S. Bagrodia, T. D. Copeland, J. L. Maller, and D. Shalloway.** 1989. Purified maturation promoting factor phosphorylates pp60$^{c\text{-}src}$ at the sites phosphorylated during fibroblast mitosis. Cell **57**:763–774.
41a. **Solomon, M.** Personal communication.
42. **Solomon, M. J., M. Glotzer, T. H. Lee, M. Philippe, and M. W. Kirschner.** 1990. Cyclin activation of p34^{cdc2}. Cell **63**:1013–1024.
43. **Stith, B. J., and J. L. Maller.** 1984. The effect of insulin on intracellular pH and ribosomal protein S6 phosphorylation in oocytes of *Xenopus laevis*. Dev. Biol. **102**:79–89.
44. **Sturgill, T. W., L. B. Ray, E. Erikson, and J. L. Maller.** 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature (London) **334**:715–718.
45. **Thomas, G., J. Marin-Pérez, M. Siegmann, and A. M. Otto.** 1982. The effect of serum, EGF, $PGF_{2\alpha}$ and insulin on S6 phosphorylation and the initiation of protein and DNA synthesis. Cell **30**:235–242.
46. **Trahey, M., and F. McCormick.** 1987. A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. Science **238**:542–545.
47. **Wahl, M. I., T. O. Daniel, and G. Carpenter.** 1988. Autophosphorylation recovery of phospholipase C activity after EGF treatment in A-431 cells. Science **241**:968–970.
48. **Ward, G. E., and M. W. Kirschner.** 1990. Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. Cell **61**:561–577.
49. **Wu, M., and J. C. Gerhart.** 1980. Partial purification and characterization of the maturation-promoting factor from eggs of *Xenopus laevis*. Dev. Biol. **79**:465–477.