Presence of cdc2+-like proteins in the preimplantation mouse embryo

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Summary

An antibody raised against a portion of the human equivalent of the yeast cdc2+ protein reacts with a 34K protein in mouse cell lines and early embryonic cells. Western blot analysis coupled with phosphatase treatment of material collected from the early preimplantation embryo has shown that the murine cdc2+ homologue does not correspond to the previously described newly synthesised proteins that are phosphorylated in a cell-cycle-dependent fashion [Howlett, 1986]. The cdc2+-like protein is converted into a slower migrating form on entry into S-phase and is further modified during G2 prior to mitosis. Studies of embryos that are held in extended periods of M-phase, i.e. unfertilised eggs or 1-cell embryos treated with nocodazole, demonstrate that the cdc2+-like protein becomes demodified in these cells.

Key words: preimplantation mouse embryo, cdc2+, murine homologies, cell cycle regulation.

Introduction

The product of the gene, p34cdc2, is a protein kinase, which plays a central role in the control of the cell cycle of Schizosaccharomyces pombe. Work with temperature-sensitive mutants has demonstrated that this protein is essential both for the transition G1–S and for entry into mitosis [Nurse, 1986; Lorinz and Reed, 1984; Simanis and Nurse, 1986]. It now appears that the mechanisms controlling the cell cycle in yeast provide a good model for more complex systems. Two recent studies have demonstrated that the human homologue of this protein is a 34K protein [Lee and Nurse, 1987; Draetta et al. 1987] which can be phosphorylated [Lee et al. 1988]. Additionally, it has been demonstrated recently that the Xenopus homologue of cdc2+ is a component of the Maturation Promoting Factor (MPF) complex [Dunphy et al. 1988a,b; Gautier et al. 1988].

It has been shown in several systems that a number of proteins undergo discrete modifications at particular points during the cell cycle [Howlett, 1986; Evans et al. 1983; Karsenti, 1987]. Detailed work on the early stages of the preimplantation mouse embryo has demonstrated that there are several proteins that are phosphorylated in a cell-cycle-dependent fashion [Howlett, 1986]. In particular a set of proteins migrating at a Mr of about 35×10^3, undergo a particularly striking series of phosphorylation and dephosphorylation events during the first and subsequent cell cycles. This protein is highly phosphorylated in unfertilised eggs arrested in M-phase and is dephosphorylated during S-phase. It is phosphorylated again on entry into first mitosis. This paper describes experiments on the mouse embryo designed to discover whether this 35K protein could be identified by an antibody raised against a portion of the human homologue of the cdc2+ protein. Using an anti-peptide antibody directed against a highly conserved region of the p34cdc2, EGVPSTAIREISLLKE, it has been shown that the 35K complex of bands does not correspond to p34cdc2. However, striking changes in the phosphorylation state of p34cdc2 occur during the first cell cycle of the mouse embryo.

Materials and methods

Recovery of embryos

3- to 4-week-old female MF1 mice (Central Animal Services, Cambridge University) were superovulated by intraperitoneal injections of 5–10i.u. of pregnant mare’s serum gonadotrophin (PMS, Intervet) and human chorionic gonadotrophin (hCG, Intervet) 44 to 48 h apart. Unfertilised eggs were recovered at 14–20 h post-hCG and freed of their cumulus cells by brief exposure to 0.1M-hyaluronidase (Sigma). In order to obtain embryos, the females were paired overnight with HC-CFLP males (Interfauna) and inspected for vaginal plugs the next day. Embryos at different stages were recovered by flushing from the oviduct at the appropriate time post hCG with Medium 2+4mgml \(^{-1}\) bovine serum albumin [M2+BSA; Fulton and Whittingham, 1978] and cultured in Medium 16 containing 4mgml \(^{-1}\) bovine serum albumin [M16+BSA; Whittingham and Wales, 1969] under oil at 37°C in 5% CO\(_2\) in air in Falcon plastic dishes.

In vitro fertilisation

In vitro fertilisation was performed as described in Howlett and Bolton, [1985].
Cytoskeletal disrupting drugs
Fertilised eggs were arrested at metaphase of their first mitosis by incubation in the microtubule-disrupting drug nocodazole (Aldrich; stock 10 mM in dimethylsulphoxide at 4°C) at a concentration of 0.5 mg ml⁻¹ in M16+BSA.

One-dimensional SDS-polyacrylamide gel electrophoresis
Embryos were cultured for 1 h in a dilution of 3 μl [³⁵S]methionine (1000–1400 Ci mmol⁻¹, Amersham International) in 50 μl M16+BSA. Embryos were washed three times with protein-free M2 and placed in 10 μl double-strength SDS sample buffer [Laemmli, 1970], boiled for 2 min and stored at -70°C. Proteins were separated on 10% SDS-polyacrylamide gels as described by Flach et al. [1982], 10 embryos being applied to each lane to facilitate comparative analysis. Following electrophoresis, gels were dried, autoradiographed and exposed for 1–3 days to preflashed Fuji X-ray film prior to autoradiography at -70°C.

Western blots
Proteins were transferred to Hybond C-extra (Amersham International) as described by Towbin et al. [1979]. After transfer, blots were shaken for 2–5 h in PBS containing 0.05% Tween 20 and 1% Marvel (blocking buffer). The affinity-purified antibody raised against cdc 2 was used at a 1:500 dilution and the blot incubated overnight in a total volume of 10 ml. The blot was then washed in blocking buffer, incubated with 125I-protein A (Amersham International) at a concentration of 3x10⁸ cts min⁻¹ ml⁻¹ in a total volume of 10 ml for 1 h, rinsed twice for 15 min in blocking buffer, before being dried prior to autoradiography. All incubations were performed at 4°C.

Potato acid phosphatase treatment
Unfertilized eggs were labelled for 1 h in medium containing [³⁵S]methionine, washed through M2+BSA, followed by M2. A control group of 10 eggs was placed directly into SDS sample buffer. Other groups of 10 eggs were placed into 5 μl double-distilled water and kept at -70°C for 30 min. Samples were then incubated at 37°C for 1 h with or without potato acid phosphatase (Sigma) at a final concentration of 5 μg ml⁻¹ (0.3 units ml⁻¹) in double-distilled water. Double-strength SDS sample buffer was added to each sample which was then treated as above for electrophoresis.

Alkaline phosphatase treatment
25 μl of 100 mM-Tris·HCl (ph 8.0), 5 mM-MgCl₂, 100 mM-NaCl were added to the sample. One microliter of a purified alkaline phosphatase preparation (Sigma P0780) was added to each sample. Control and treated samples were incubated for 15 min at 37°C. To some samples, 100 mM-p-nitrophenyl phosphate was also added in order to confirm that the observed effects were caused by dephosphorylation and not by proteolytic artifact.

Growth and fractionation of L-cells and HeLa cells
Mouse fibroblast-like cells (strain L, clone 292) were grown to stationary phase, at which point most cells become arrested in G₀ prior to S-phase. Cytoplasmic extracts were prepared from concentrated cells, after hypertonic swelling, by mechanical breakage using a dounce homogenizer. Postnuclear supernatants were prepared by spinning at 3000 g for 5 min, a postmitochondrial fraction was then prepared from this supernatant by centrifugation for a further 10 min at 10000 g. This postmitochondrial supernatant had a protein concentration of 0.5 mg ml⁻¹. Human HeLa cell extracts were prepared in a similar way.

Antibody generation
The antibody used throughout this work was raised against a 15 amino acid peptide, represented using the single amino acid letter code by EGVSTAIRHESSLKE. This region of the protein is conserved between humans, S. pombe and S. cerevisiae and spans amino acids 42–57 in the human homologue of cdc2⁰. Peptide conjugation, antibody production and affinity purification of this antibody were performed exactly as described in Simanis and Nurse [1986].

Visual identification of fertilised eggs
4–6 h after fertilisation, unfertilised and fertilised eggs could be distinguished, by observation under a Wild dissecting microscope at a magnification of x60, by the appearance of the male and female pronuclei at the periphery of the egg. These pronuclei migrate centrally at 11–12 h postinsemination prior to syngamy and first mitosis.

Results
The 35K complex does not correspond to p34cdc2
The newly synthesised 35K protein is modified by phosphorylation during the cell cycle, and as judged by SDS gel electrophoresis has a relative molecular mass of about 35x10⁴. The phosphorylation occurs at sites that are sensitive to potato acid phosphatase [Howlett, 1986], and may also contain some carbohydrate moieties [Van Blerkom, 1981]. In this study, we used SDS gel analysis coupled with phosphatase treatment and Western blotting to determine if the cdc2⁰ homologue in mice possessed similar characteristics.

500 unfertilised mouse eggs were collected, of which 480 were dissolved in SDS sample buffer and the remainder were labelled with [³⁵S]methionine. This labelled sample was then divided into two equal parts and, after lysis in water, the radiolabelled material was incubated either with or without phosphatase. 2 μl samples of the postmitochondrial supernatants from L-cells and HeLa cells were also incubated with water alone. All samples were then subjected to electrophoresis on a 10% polyacrylamide gel. The section of the gel containing the in vivo labelled material was fixed and dried, while the section containing the L-cell and HeLa cell extracts and the unfertilised eggs was transferred to nitrocellulose, and the blot incubated with the antibody known to recognise the human homologue of cdc2⁰. Radiolabelled standards were included in both the in vivo labelled portion of the gel and the Western blot, enabling easy alignment of the resulting autoradiograms, which are shown in Fig. 1.

From the results presented in this figure several conclusions can be drawn. First, material reacting with the antibody directed towards the cdc2⁰-like protein migrates slightly faster than the 35K protein, which labels strongly with [³⁵S]methionine and is indicated by arrows (compare tracks 1 and 3–5). Moreover, although phosphatase treatment results in a shift in the radiolabelled 35K protein (compare tracks 1 and 2), even after digestion this protein does not migrate at the
same position as the material reacting with the anti-cdc2 antibody on Western blotting. Lastly, it was noticed that the antibody recognised a closely spaced doublet in L-cell and HeLa cell extracts, but material from unfertilised eggs seemed to have only the lower of these two bands (longer exposure of the autoradiogram shown in Fig. 1 did not result in the appearance of an additional upper band).

The cdc2+ -like protein is modified during interphase

These observations seem to suggest that the p34\(^{cde2}\) protein existed in two forms in unsynchronised cultured cells from which the L-cell and HeLa extracts were made, whereas for the unfertilised eggs, which are arrested in second meiotic metaphase, only the lower of the two bands was present. To test the hypothesis that the M-phase cells contained a dephosphorylated form of p34\(^{cde2}\), we collected 1000 unfertilised eggs and 1000 fertilised eggs in interphase i.e. 11.5—12 h postovulation. Fertilised and unfertilised eggs can be distinguished easily at this stage (see Fig. 6). 20 fertilised and 20 unfertilised eggs were sampled and labelled with \(^{35}\)S\text{methionine}. Labelled and unlabelled samples were then lysed, divided equally and incubated in water alone or in water plus phosphatase. The samples were then dissolved in SDS gel sample buffer, and analysed on a 10% polyacrylamide gel. The portion of the gel containing radiolabelled samples was fixed and dried prior to autoradiography, while the remainder was Western blotted. The resulting autoradiogram is shown in Fig. 2. The patterns in tracks 2—5 are those that might be expected from previous experiments [Howlett, 1986]. Thus, the highly phosphorylated form of the 35K protein present in unfertilised eggs is sensitive to phosphatase (compare tracks 1 and 2), whereas the

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**Fig. 1.** Comparison of relative molecular mass of 35K complex with the cdc2+ homologue in mice. Tracks 1 and 2 in vivo labelled pattern of unfertilised eggs incubated without (1) or with (2) potato acid phosphatase: tracks 3, 4 and 5 Western blot analysis of cdc2+ like material: in track 3, L cell extract, track 4, HeLa cell extract and track 5 unfertilised eggs. Position of upper and lower bands of 35K complex indicated by arrows.

**Fig. 2.** Comparison of behaviour of cdc2+ homologue with 35K complex during M-phase and S-phase. Tracks 1 and 2: \(^{35}\)S\text{methionine}-labelled pattern of unfertilised eggs incubated alone (track 1), or with potato acid phosphatase (track 2). Tracks 3 and 4: \(^{35}\)S\text{methionine}-labelled S phase embryos incubated alone (track 3), or with potato acid phosphatase (track 4). Tracks 5, 6, 7 and 8 represent the results of a Western blot analysis using the antibody to cdc2+, 250 unfertilised eggs (tracks 5 and 6) or S-phase zygotes (tracks 7 and 8) were incubated alone (tracks 5 and 7) or with potato acid phosphatase (tracks 6 and 8). Blots were incubated with antibody overnight at 4 °C and any bound antibody visualised by \(^{125}\)I-protein-A binding.
The murine homologue of cdc2 + becomes modified on entering S-phase and again at G2

We were interested to discover the exact point during the first cell cycle at which the modification resulting in a slower migrating form of p34cdc2 occurred. The first cell cycle of mouse development is an ideal system in which to study this phenomenon since a high degree of synchrony can be achieved by in vitro fertilisation, and also because the exact onset of S-phase has been described accurately in terms of morphology and of in vitro [35S]methionine-labelling patterns [Howlett and Bolton, 1985].

Embryos were fertilised in vitro, and samples of 250 synchronised embryos were collected at 4h intervals during the first cell cycle. 20 of these embryos were labelled with [35S]methionine and the remainder used for Western blotting. The result of such an experiment is shown in Fig. 4. The protein synthetic profiles are exactly those expected from previous work [Howlett and Bolton, 1985; Howlett, 1986].

The corresponding Western blots arising from such a timed study are shown in tracks 7–12. These data demonstrate that as the embryos enter S-phase, i.e. 12 h postfertilisation (tracks 4 and 10), the murine version of the cdc2 + -like protein is modified into a slower migrating form (track 10). Track 6 shows the 35S-biosynthetic labelling pattern typical of cells held in M-phase by nocodazole; the corresponding panel from the Western blot (track 12) shows that, in this prolonged state of M-phase, the cdc2 + homologue in mice exists as a faster migrating form. Track 13 shows the pattern observed when embryos were collected in very late G2/M just prior to mitosis. From this track it can be seen that the cdc2 + homologue in mice is modified on entry into S-phase, and it also appears to be converted partially into an even slower migrating form in late G2/M, but when the cells are held in a prolonged M-phase then the cdc2-like protein is converted into the faster moving form which is seen in G1.

The murine homologue of cdc2 + is phosphorylated on entry into S phase

To investigate the nature of the modification to the cdc2 + homologue occurring during S-phase, embryos were collected either as unfertilised eggs or during S-phase. A small number were labelled with [35S]meth-
ionine and the remainder were reserved for Western blotting. At each stage the samples, both labelled and unlabelled, were divided into three equal parts and incubated with buffer alone, buffer plus alkaline phosphatase, or with alkaline phosphatase plus p-nitrophenol phosphate [exactly as described in Draetta and Beach, 1988]. The results of such an experiment are shown in Fig. 5. Treatment with alkaline phosphatase causes an alteration in the mobility of the 35K complex present in unfertilised M-phase eggs (track 2), but no alteration to the mobility of the 35K complex during S-phase (track 5). The opposite appears to be the case for the cdc2+ homologue i.e. a small shift is observed (track 11) when S-phase material but not M-phase material (track 8) is digested with the enzyme: the effect was prevented when p-nitrophenol phosphate was added to inhibit the alkaline phosphatase (compare tracks 10 and 12).

**Discussion**

The data presented in this paper clearly demonstrate that the 35K protein described previously [Howlett, 1986] is not the same as the cdc2+ homologue in mice, because it migrates in a different position on polyacryl-
amide gels and is sensitive to acid phosphatase treatment. Furthermore, the cdc2\(^+\) protein is modified during the cell cycle in the opposite way to the 35K complex. In unfertilised eggs, the 35K protein is highly phosphorylated and migrates more slowly than during interphase, whereas the cdc2\(^+\) protein migrates more rapidly in the unfertilised egg than during S/G2 when it becomes phosphorylated. Carefully timed studies through the first cell cycle have demonstrated that, whilst the 35K protein is dephosphorylated during G1 and the transition to S-phase, the cdc2\(^+\) homologue is first phosphorylated at the G1 to S transition and is modified further during G2 prior to mitosis. Additionally, these studies have demonstrated that embryos held in M-phase, either naturally as in the case of unfertilised eggs or artificially when late G2 embryos are incubated in nocodazole, contain the more rapidly migrating form of the cdc2\(^+\) protein, whilst the 35K protein remains phosphorylated under these conditions. From the results of the phosphatase experiments shown in Fig. 5, it seems probable that the faster migrating form of the cdc2\(^+\) protein seen during elongated M-phase represents the dephosphorylated form of the cdc2\(^+\) homologue. Thus the dephosphorylation of cdc2\(^+\), unlike that of the 35K protein, does not seem to require transition from metaphase to anaphase. A schematic representation of the behaviour of the cdc2\(^+\) and 35K proteins is shown in Fig. 6.

If the overall phosphorylation state of the 35K and cdc2\(^+\) proteins is important in controlling their activities, then the data presented here would suggest that the two different functions. The cdc2\(^+\) protein is phosphorylated at G1 to S and then at late G2 to M transitions. It is known that, in yeast, cdc2\(^+\) is implicated in control at two points in the cell cycle, once at 'start' and again at the transition for completion of mitosis [Nurse, 1986]. Cdc2 can act as a kinase, it is known to phosphorylate histone 1 and to be a component of MPF [Dunphy et al. 1988b; Gautier et al. 1988], which might reflect roles in both S and G2/M-phases. The role of the 35K complex is unknown, but since it shows, with several other cell cycle proteins, an increased phosphorylation during M-phase [Karsenti et al. 1987], perhaps it represents one of a set of endogenous phosphoproteins that are substrates for a single driving kinase activity such as might perhaps be provided by cdc2\(^+\). We are currently attempting to manipulate the activity of the cdc2\(^+\) protein to determine the impact on both progression through the cell cycle and the modification of the 35K complex. Such experiments may reveal whether the cdc2\(^+\) protein drives the changes detected in the 35K complex.

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References


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