From Cdc2 to Cdk1: when did the cell cycle kinase join its cyclin partner?

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Summary

The idea that Cdc2 and cyclins play a key role in the control of the G2/M transition of the cell cycle came largely from genetic analysis of fission yeast and physiological studies of clam, frog, sea urchin and starfish eggs and oocytes. However, it took a long time to realise that Cdc2 and cyclins form a stoichiometric complex and that a cyclin subunit is necessary for the Cdc2 subunit to gain its protein kinase activity.

Cyclins were first recognized as proteins whose abundance oscillates during the early cell cycles of marine invertebrate eggs and their connection with MPF (maturation-promoting factor), the entity defined in frog and starfish oocytes whose activity controls entry into M phase, was far from clear at first. Indeed, it was a long time before MPF was shown to be a protein kinase, and direct proof that MPF is a heterodimer comprising one molecule of cyclin and one molecule of Cdc2 was finally obtained only when the Cdc2-associated component of purified starfish MPF was sequenced and found to be cyclin B. When this fundamental discovery was confirmed in vertebrates and mammalian members of the Cdc2 family were also shown to bind cyclins, Cdc2 became Cdk1, the first cyclin-dependent protein kinase.

Key words: Cell cycle, CDK, Cyclin, MPF, Oocyte, Protein kinase

Introduction

The two major processes common to all cell cycles are S phase, when chromosomes are replicated, and M phase, when the replicated chromosomes are segregated into two daughter cells. In most cell cycles, an interval of time, G1 phase, separates the previous cell division from the beginning of the next S phase. It is now firmly established that progression of the cell cycle – that is, transitions between one phase of the cycle and the next – are controlled by cyclin-dependent kinases (CDKs). This concept of a direct association between a cyclin subunit and a cell cycle kinase subunit (Cdc2) emerged from analysis of the G2/M transition. Fission yeast mutants that are prematurely advanced into mitosis and thus enter prophase at reduced size focused attention on Cdc2, but gave no clues about cyclins. In budding yeast, which are less favourable for investigation of the G2/M transition because growth occurs mainly in G1 phase, attention focused largely on the G1/S transition. Thus, paradoxically, the wealth of information about cell cycle genes in *S. cerevisiae* provided only a limited number of clues at first in putting cyclins together with CDKs. In contrast, efforts to purify and identify the mysterious MPF (Masui and Markert, 1971) were of paramount importance on the way to our present understanding of G2/M and, by extension, other transitions of the cell cycle. Here we look back to this heroic age and try to establish precisely when and how Cdc2 became Cdk1.

Cyclins as MPF activators

Fully grown oocytes stimulated with species-specific signals (sometimes hormones, sometimes fertilization) are released from G2 arrest and enter M phase. At this time, their cytoplasm has gained MPF activity and, even in the absence of protein synthesis (Wasserman and Masui, 1975), it drives recipient oocytes into M phase when transferred into G2-arrested oocytes. One of the most dramatic events that follow hormonal stimulation in starfish and amphibian oocytes is protein phosphorylation, which is associated with increased protein kinase activities (Guerrier et al., 1977; Maller et al., 1977). In 1983 the extent of protein phosphorylation in starfish oocytes was shown to oscillate simultaneously with activity of MPF during meiotic maturation, high and low levels of protein phosphorylation being associated with high and low levels of MPF activities, respectively (Dorée et al., 1983). The same year, Wagenaar analyzed the timing of synthesis of proteins required for mitosis in the early cell cycles of sea urchin embryos (Wagenaar, 1983), extending previous reports that puromycin added at the time of fertilization prevents the first division (Hultin, 1961). This demonstrated that protein synthesis is required at each cell cycle for chromosome condensation and nuclear envelope breakdown and thus for the G2/M phase transition to occur. It was also found that protein synthesis is required in *Xenopus* oocytes for reappearance of MPF activity in the second meiotic cell cycle or the early embryo after fertilization and parthenogenetic activation (Gerhart et al., 1984). The simultaneous oscillation of MPF activity and that of a major cAMP-independent histone kinase were then reported to depend on protein synthesis and protein degradation during meiotic maturation of starfish oocytes, which led to the provocative proposal that the irreversible process of proteolysis might be involved in periodic inactivation of a major cycling kinase and in the drop of MPF activity at exit from M phase (Picard et al., 1985). Obviously, such features were reminiscent of cyclins, which had been identified in 1983 as proteins degraded at the end of each M-phase cycle in the early sea urchin embryo (Evans et al., 1983).

The view that mitotic cyclins are in some way linked with oscillations in MPF and mitotic kinase activities was strengthened when it was shown that expression of clam cyclin A releases *Xenopus* oocytes from G2 arrest and induces meiotic M phase in the absence of hormonal stimulation (Swenson et al., 1986). Two years later, Murray and Kirschner succeeded in producing 'cycling' extracts of frog eggs that perform multiple cell cycles in vitro (Murray and Kirschner, 1989). They showed that, after destruction of endogenous cellular messenger RNAs, which arrests the extracts in interphase, addition of exogenous cyclin mRNA is sufficient to produce multiple cell cycles. Moreover, they observed that the newly synthesized cyclin is degraded at the end of each mitosis (Murray and Kirschner, 1989). The view that the synthesis of cyclin B is necessary for mitotic cell cycles in cleaving *Xenopus* embryos became firmly established when Minshull et al., who identified two cyclins as major translation products in cell-free extracts, reported that antisense-mediated destruction of these mRNAs blocks entry into mitosis (Minshull et al., 1989a).

Together, these findings demonstrated that cyclin is necessary for the appearance of MPF and that MPF disappears because of destruction of cyclin. Furthermore, they strongly suggested that the activity of MPF is intimately linked to a mitotic kinase activity whose oscillations share with MPF the same requirements for protein synthesis and degradation.

Cdc2 as a component of MPF and the major mitotic kinase

Although MPF was first described in 1971, extensive purification of MPF from *Xenopus* eggs was not reported until 1988, when Maller and co-workers used a cell-free assay to monitor MPF activity (Maller et al., 1977). For reasons that are still unclear, this required ATP-γS, a reagent used to thiophosphorylate proteins and protect them against phosphatases. The most highly purified preparation contained two major proteins of mass 32 kDa and 45 kDa, and displayed protein kinase activity (Lohka et al., 1988). The major 32 kDa protein could be the *Xenopus* homolog of Cdc2, a protein kinase required for mitosis in fission yeast, which had been previously shown by functional complementation of mutants to be conserved from yeast to humans (Lee and Nurse, 1987). Consistent with this view, Maller's group reported that an antibody directed against a conserved 16-residue sequence of Cdc2 (the so-called PSTAIRE antibody) could deplete the purified preparation of its MPF activity and immunoprecipitate the MPF-associated protein kinase together with the 32 kDa component (Gautier et al., 1988). The 45 kDa component was not identified. Using an affinity column containing the product of the *Suc1* gene, p13, which had been shown to interact physically with Cdc2 in fission yeast, Dunphy and Newport could also deplete MPF activity from cell-free extracts prepared from *Xenopus* eggs (Dunphy and Newport, 1989). Besides Cdc2, also identified by immunoblotting, a few other proteins were retained on the $p13^{suc1}$ column, including an unidentified 42 kDa protein. These results strongly suggested not only that the Cdc2 kinase was required to maintain MPF activity but that MPF was in fact simply Cdc2.

In parallel with the investigations mentioned above, two other groups used in vitro phosphorylation of histones as an assay to purify by conventional column chromatography the major mitotic kinase from starfish oocytes (Arion et al., 1988; Labbé et al., 1988). Using PSTAIRE immunoblotting and/or binding to $p13^{suc1}$, they concluded that it was the Cdc2 kinase. Since Cdc2 appeared to be a component of *Xenopus* MPF, Arion et al. suggested that Cdc2 kinase and MPF could be the same entity, although they did not address this question experimentally. However, this interpretation was difficult to reconcile with the fact that, although enucleated starfish oocytes readily activate Cdc2 kinase in response to hormonal stimulation, they do not produce transferable MPF activity (Kishimoto et al., 1981; Picard et al., 1984; Picard et al., 1988).

From Cdc2 to Cdk1

Cdc2 and cyclins as interacting proteins

Using crude homogenates from early clam embryos, it was found that cyclins co-immunoprecipitate with Cdc2 kinase activity (Draetta et al., 1989). Cdc13, the major cyclin in fission yeast, was next shown to co-precipitate with Cdc2 and to dissociate from Cdc2 at the restrictive temperature in *cdc13-117* thermosensitive mutants (Booher et al., 1989). This suggested that cyclins could be subunits of Cdc2 kinase, even though Cdc2 retained H1 kinase activity at the restrictive temperature (Moreno et al., 1989). The alternative possibility was that cyclins are mitotic substrates of Cdc2, and in fact they were shown to undergo phosphorylation when [γ-^{32}P]ATP was added to materials retained on $p13^{suc1}$ beads. Yet against this was another study using early sea urchin embryos, which showed that cyclin B co-purifies with Cdc2 kinase along several steps of column chromatography (Meijer et al., 1989). This favoured the first interpretation.

In the same year, it was reported that associated proteins could apparently be removed from Cdc2 without loss of its histone kinase activity after extensive purification from starfish oocytes (Labbé et al., 1989a). Although this suggested no absolute requirement for an activator protein associated with Cdc2, cyclin could have undergone proteolysis during the lengthy purification procedure, so that no fragment of consistent size peaking with Cdc2 kinase activity would have been detected even when consecutive fractions were silver-stained after analysis by SDS-PAGE in the final preparation. Nor was autophosphorylation of any polypeptide detected in the final peak of activity when the kinase was incubated with [γ-^{32}P]ATP; yet, a cyclin fragment lacking the phosphorylation sites could still have been associated with Cdc2. Microinjection of this highly purified Cdc2 kinase readily released oocytes from G2 arrest in a variety of species. Surprisingly, it did so even in starfish, even though the native Cdc2 kinase is not sufficient (in the absence of nuclear material) to induce germinal vesicle breakdown in non-hormone-stimulated starfish oocytes.

At that time, only a few protein kinases that contain regulatory subunits had been identified, and the paradigm for such kinases was the cAMP-dependent protein kinase (PKA), whose regulatory subunit had been shown to negatively control the activity of the catalytic subunit. Thus, even for people convinced that MPF was indeed a protein kinase, the positive role that cyclin seemed to have in regulation of MPF activity did not imply that it was necessarily a subunit of this kinase. In fact, no phenotype was associated with overexpression of Cdc13 in fission yeast (Booher and Beach, 1988).

The MPF kinase was, rather, believed to be kept inactive through association with a hypothetical inhibitory subunit:

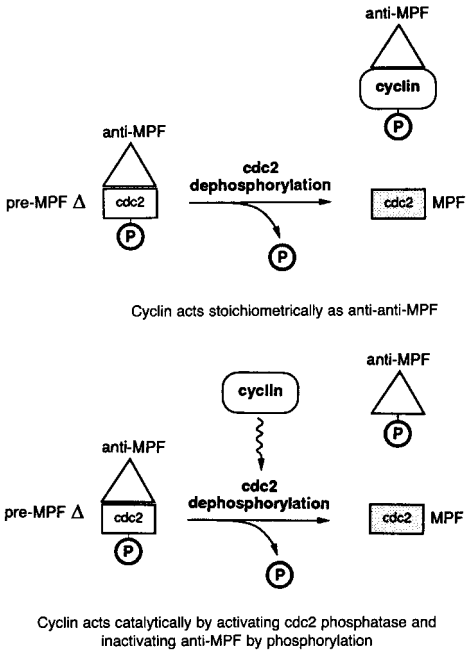

Fig. 1. Two early models to explain how cyclin activates MPF (Minshull et al., 1989b), published a few months before MPF was identified as a stoichiometric complex comprising one molecule of Cdc2 and one molecule of cyclin B. The upper cartoon shows cyclin as binding and thereby removing a hypothetical anti-MPF subunit, thus activating Cdc2. According to this diagram, anti-MPF acts stoichiometrically, but both anti-MPF and cyclin might act catalytically, for example to alter the phosphorylation state of Cdc2. In the lower model, cyclin modifies the hypothetical inhibitor, which allows Cdc2 dephosphorylation.

binding of the cyclin was proposed to dissociate the inhibitory subunit (anti-MPF) and activate the kinase (Minshull et al., 1989b) (Fig. 1). In agreement with this view, an anti-MPF entity called INH (for inhibitor of MPF amplification) had been characterized in *Xenopus* oocytes (Cyert and Kirschner, 1988). In addition, negative control of Cdc2 kinase through phosphorylation had been demonstrated in both starfish and *Xenopus* oocytes (Labbé et al., 1989a; Dunphy and Newport, 1989; Gautier et al., 1989), which was consistent with genetic studies in fission yeast demonstrating that Wee1 kinase negatively controls Cdc2 kinase (Russell and Nurse, 1987). Cyclin could thus control MPF activation by triggering dephosphorylation of Cdc2 [that cyclin is already associated with Cdc2 prior to its dephosphorylation by Cdc25c was demonstrated only two years later (Gautier and Maller, 1991; Strausfeld et al., 1991).

Identification of starfish MPF as a cyclin-B–Cdc2 heterodimer

Labbé et al. provided decisive evidence that cyclin B is a genuine subunit of Cdc2 kinase when they reported purification to homogeneity of the M-phase-specific kinase from starfish oocytes at first meiotic metaphase, using a rapid three-step procedure based on affinity chromatography on immobilized yeast protein $p13^{suc1}$ (Labbé et al., 1989b). Besides $p34^{cdc2}$, the final peak of H1 kinase activity contained only one other polypeptide, which had an apparent molecular weight of 47 kDa and was identified as starfish cyclin B by direct microsequencing.

When the final preparation was incubated with [γ-^{32}P]ATP, cyclin B became extensively labeled; Cdc2 was labelled to a much lesser extent, if at all. All the active fractions contained both Cdc2 and cyclin B, and the latter was not detected outside the peak of activity. To strengthen the view that both proteins were associated in a complex, Labbé et al. separated Cdc2 from cyclin B by using an HPLC reverse-phase column developed with an acetonitrile gradient (Labbé et al., 1989b). Quantifying the relative absorbance of each protein by monitoring absorbance at 220 nm, they observed a constant 1:1 stoichiometry of Cdc2 and cyclin B in independent preparations and in consecutive fractions throughout the final peak of kinase activity. These results demonstrated that the purified kinase is a heterodimer containing one molecule of Cdc2 and one molecule of cyclin B.

The purified kinase readily induced germinal vesicle breakdown and meiotic maturation when injected into *Xenopus* oocytes, which was consistent with the reported lack of zoological specificity of MPF (Kishimoto et al., 1982). Moreover, it was later shown to induce meiotic maturation in starfish oocytes only when microinjected into the nucleus. When injected into the cytoplasm, it underwent rapid inactivation, as does the native kinase when transferred from enucleated donor oocytes (Picard et al., 1991). Thus the heterodimeric Cdc2 kinase behaved differently from the earlier preparation (Labbé et al., 1989a) in which Cdc2 was the only major protein.

When antibodies against recombinant starfish cyclin B became available (Strausfeld et al., 1991), it could be shown that cyclin B undergoes proteolysis during the gel filtration step of the first purification procedure, generating numerous polypeptide fragments, none of which is present in sufficient amounts to be detected by silver staining in the final preparation. At least part of the cyclin box escapes proteolysis, owing to its secondary structure. Since only the N-terminal domain of cyclin contains phosphorylation sites, the final preparation failed to autophosphorylate Cdc2-bound cyclin fragments. Moreover, because this truncated cyclin lacks the N-terminal CRS (cytoplasmic retention signal) and NES (nuclear exclusion signal) of cyclin B, the kinase complex rapidly translocates into the nucleus of recipient oocytes and escapes cytoplasmic inactivation when microinjected into G2-arrested oocytes. This probably accounts for its paradoxically high MPF activity in starfish.

Towards a CDK family of protein kinases

One year later, identification of cyclin-B–Cdc2 as MPF was extended to *Xenopus* oocytes: using specific antibodies against *Xenopus* cyclins, Gautier et al. showed that their highly purified MPF preparation prepared from oocytes at second metaphase (see above) contained a mixture of cyclin-B1–Cdc2 and cyclin-B2–Cdc2 (Gautier et al., 1990). No cyclin-A–Cdc2 was detected, even though oocytes produce cyclin A in the second meiotic cell cycle. In 1990 and 1991, close relatives of Cdc2 were identified in *Drosophila* and *Xenopus* (Lehner and O'Farrell, 1990; Paris et al., 1991). Neither of these could rescue cell cycle arrest caused by mutations in *cdc2* or *CDC28* in yeasts, but Fang and Newport showed that selective depletion of the *Xenopus* cdc2-related protein (known as Eg-1 at that time) can suppress DNA replication in *Xenopus* egg extracts, a finding at variance with depletion of Cdc2 (Fang and Newport, 1991). Moreover, it was shown that a close relative of Eg-1 in humans can rescue G1 arrest in *CDC28* budding yeast mutants but not

G2 arrest in mutants of fission yeast *cdc2* (Ninomiya-Tsugi et al., 1991; Elledge and Spottswood, 1991). This demonstrated that both Cdc2 relatives have different functions in the control of the cell cycle. Both were shown to bind to cyclin A. When it became evident that mammalian Cdc2 homologs can also bind to cyclins (Pines and Hunter, 1990), a new convention for naming these kinases was established by consensus at the Cold Spring Harbor Symposium on the Cell Cycle in 1991: kinases that are associated with cyclins would be called 'cyclin-dependent kinases', or CDKs. And so Cdc2 became Cdk1…

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