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COMMENTARY

M-phase-promoting factor activation

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Introduction

Two decades ago, Hartwell and co-workers isolated a number of cell division cycle (*cdc*) mutants in *Saccharomyces cerevisiae* on the basis of their arrest at specific, morphologically distinguishable points in the cell cycle. These temperature-sensitive mutants provided the first identification and temporal ordering of genes required for progress through the cell cycle, and permitted the first molecular definition of a cell cycle restriction point, *START*, passage through which requires the function of the *CDC28* gene. Interestingly, *CDC28* was found to be required not only for leaving stationary phase and commencing DNA replication, but also for the events of nuclear division (Hartwell et al., 1974; Hartwell and Weinert, 1989; Reed et al., 1985). Similarly, in the fission yeast *Schizosaccharomyces pombe*, the homologous *cdc2* gene was found to be necessary for executing both the G_1/S and G_2/M transitions (Nurse et al., 1976; Nurse and Bisset, 1981; Beach et al., 1981). *CDC28/cdc2*, and their homologs in species spanning the plant and animal kingdoms, encode protein kinases of approximately 34 kDa (Hindley and Phear, 1984; Reed et al., 1985; Simanis and Nurse, 1986), referred to hereafter simply as p34. In animal cells, increasingly sophisticated molecular techniques have led to the identification of a family of p34-related genes (Pines and Hunter, 1991), some of which are clearly distinct from *CDC28* or *cdc2* and may mediate some of the G_1/S functions previously attributed exclusively to *CDC28/cdc2* (Elledge and Spottswood, 1991; Fang and Newport, 1991; Koff et al., 1991; Lehner and O'Farrell, 1990; Paris et al., 1991; Tsai et al., 1991).

Cyclin-dependent protein kinases and MPF

Although the effects of p34 are cell-cycle-specific, its levels undergo no dramatic fluctuation during the cell cycle. Instead, modulation of p34 activity occurs through association with regulatory subunits, called cyclins, which appear and disappear in orderly fashion during the course of the cell cycle. (Properties of the different cyclins are outlined by Hunter and Pines (1991).) For example, the *CLN1* and *CLN2* genes of *S. cerevisiae* are expressed in G_1 during growth in nutrient-rich medium, but transcription ceases in the presence of mating factor (Nash et al., 1988; Hadwiger et al., 1989; Wittenberg et al., 1990). The murine macrophage *CYL* genes are specifically induced in response to colony-stimulating factor 1, but the D-type cyclins that they encode disappear after withdrawal of the stimulus, once cells leave G_1 and enter S phase (Matsushime et al., 1991; cf., however, Motokura et al., 1991). Other G_1 cyclins, identified either by their ability to complement budding yeast *cln* mutants, by polymerase chain reaction (PCR) or by hybridization, include cyclins C and E (Koff et al., 1991; Léopold and O'Farrell, 1991; Lew et al., 1991). Cyclin B is the predominant G_2/M cyclin, and is rapidly proteolyzed at the onset of anaphase (Evans et al., 1983; Booher and Beach, 1987; Standart et al., 1987; Luca and Ruderman, 1989; Minshull et al., 1989; Pines and Hunter, 1989; Westendorf et al., 1989; Ghiara et al., 1991; Surana et al., 1991). Cyclin A, found only in animal cells, is implicated by genetic analysis in *Drosophila* in control of mitosis (Lehner and O'Farrell, 1989). However, its association with E1A (Giordano et al., 1989; Pines and Hunter, 1990) and retinoblastoma gene product (Rb) (Bandara et al., 1991; Mudryj et al., 1991) suggests a role earlier in the cell cycle. In fact, in human cells, the kinase activity associated with cyclin A seems to be maximal in S and G_2 (Pines and Hunter, 1990).

Cyclin and p34 combine to form a holoenzyme complex, giving rise to the designation cyclin-dependent protein kinase (CDK) to refer to all members of the p34 protein kinase family. The effects of CDKs are mediated through cell-cycle-specific phosphorylations of key substrates. For example, in *S. cerevisiae* different isoforms of cyclin B can impart different substrate specificites to p34 (Surana et al., 1991). Specificity may at least in part be determined by substrate accessibility, since in *S. pombe* subcellular localization of p34 requires an intact cyclin B gene (Hagan et al., 1988; Alfa et al., 1989; Booher et al., 1989), and might thus explain why it has been difficult to demonstrate differences in substrate preference in vitro among

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various p34-cyclin combinations (Draetta et al., 1989; Minshull et al., 1990; Feuerstein, 1991). Not surprisingly, inappropriate expression of cyclins short-circuits normal cell-cycle control: dominant, hyperstable *S. cerevisiae CLN* mutants permit cells to divide in the presence of mating factor or in the absence of nutrients (Cross, 1988; Hadwiger et al., 1989; Nash et al., 1988), microinjection of cyclin A or cyclin B message into interphase-arrested oocytes triggers the onset of metaphase (Swenson et al., 1986; Westendorf et al., 1989), while overexpression of cyclin B prevents cells from exiting mitosis (Murray et al., 1989; Ghiara et al., 1991). Tight control of cyclin levels is accomplished by regulation of both transcription and proteolysis (summarized by Hunter and Pines, 1991) as well as specific inactivation (Chang and Herskowitz, 1990; Elion et al., 1990). The possibility that deregulation of cyclin levels may contribute to oncogenesis has recently been discussed (Hunter, 1991; Hunter and Pines, 1991).

p34 and cyclin B are the principal subunits of M-phase-promoting factor (MPF), first purified from *Xenopus* on the basis of its ability to induce mitotic events in cell-free extracts (Lohka et al., 1988), and distinguished biochemically by its ability to phosphorylate histone H1 on specific sites (Woodford and Pardee, 1986; Langan et al., 1989). (The p34-cyclin B MPF complex has been the subject of several recent reviews: Dorée (1990); Maller (1990); Nurse (1990).) MPF purified from a variety of sources shows surprising variability in size, even when prepared from the same species, ranging from 34 kDa to much larger forms (Table 1). This implies that additional proteins may associate with the p34-cyclin B complex, perhaps as tightly bound substrates or as additional regulatory components. Several high molecular weight preparations of MPF contain a 67 kDa component (Table 1). A protein with a similar molecular weight (p65) is recognized by antibodies raised against mitosis-specific protein kinases from human cells (Meikrantz et al., 1990), with immunoreactive analogs detected in *S. cerevisiae*, *Xenopus*, starfish, *Drosophila* and mouse (L. Wilson, M. Lohka, L. Meijer, D. Gilmour, A. Nordin, W. Meikrantz and R.A. Schlegel, personal communications and unpublished observations). Although p65 is present throughout the cell cycle in human cells, at mitosis it forms a disulfide-linked homodimer of 130 kDa that co-precipitates with p34 and cyclin B (Meikrantz et al., 1990). In addition, anti-p65 IgG depletes mitotic extracts of H1 kinase activity, and p65 can be cleared from mitotic extracts using p13, a CDK-specific ligand encoded by the *S. pombe suc1* gene (Brizuela et al., 1987), coupled to Sepharose beads (Meikrantz et al., 1991b). Interestingly, p65 interacts in a mitosis-specific manner with at least one other member of the CDK family, PSK-J3 (Meikrantz et al., 1990).

The appearance of p65/p67 in some MPF preparations but not in others suggests that it may only transiently associate with the p34-cyclin B complex. Alternatively, subunits of the MPF complex may be in dynamic equilibrium, with particular isolation conditions selectively stabilizing one form of the complex and not others. The concept of an equilibrium between multiple forms of MPF existing at mitosis is supported by an early result of Wasserman and Masui (1976): when unfractionated *Xenopus* ooplasm was separated by sucrose gradient ultracentrifugation, three separate peaks of MPF activity were found. In *S. cerevisiae*, the multiple high molecular weight cdc28-containing complexes found are thought to represent different stages in its activation (Wittenberg and Reed, 1988).

Table 1. *Composition of mitosis-specific H1 kinase and MPF*

Source	Size (M_r)[a]	Subunit composition[b]	H1 kinase activity (nmol P_i/mg per min)
Xenopus[c]	200,000	p34 Cyclin B Other?	270
	50,000	p34 Cyclin B	nd[d]
CHO[e]	nd	35,000 60,000 67,000	100-300
Novikoff rat hepatoma[f]	nd	p34 Other?	500
Starfish[g]	40,000	p34	500
	nd	p34 Cyclin B	8,000
Sea urchin[h]	200,000	p34 Cyclin B 67,000	nd
HeLa cells[i]	220,000	p34 Cyclin B Other?	~100[j]

[a]Relative molecular mass as determined by gel filtration chromatography or glycerol gradient centrifugation.
[b]p34 and cyclin B are designated as such where they have been identified; other components are identified by their relative migration on SDS-PAGE.
[c]From mature ooplasm. Data for the 200×10^3 M_r (multimeric) form are those of Lohka et al. (1988). Data for the $45\text{-}55\times10^3$ M_r (monomeric) form are those of Erikson and Maller (1989).
[d]nd, no data available.
[e]Isolated from tissue culture cells synchronized in G_2/M on the basis of H1 kinase activity (Woodford and Pardee, 1986).
[f]Isolated on the basis of H1 kinase activity (Langan et al. 1989).
[g]From mature ooplasm. Monomeric preparation: purified on the basis of H1 kinase activity alone (Labbé et al. 1988); dimeric preparation: purification by H1 kinase and MPF activity (Labbé et al. 1989).
[h]Purification by H1 kinase activity and MPF activity from fertilized eggs (Arion et al. 1988).
[i]Isolated on the basis of cyclin B autophosphorylation from cells partially synchronized in M phase (Brizuela et al. 1989).
[j]Estimated from the published data (given in cts/min per mg).

Activation of MPF

Formation of MPF is not a simple matter of accumulating sufficient amounts of cyclin B during interphase to reach a certain threshhold level at G_2/M (Felix et al., 1989, 1990; Minshull et al., 1989; Murray and Kirschner, 1989; Nurse, 1990). In order to prevent

mitosis from occuring before DNA synthesis and repair are completed (Hartwell and Weinert, 1989; Dasso and Newport, 1990; Broek et al., 1991; Enoch and Nurse, 1990, 1991), p34 and cyclin B are sequestered in an inactive pre-MPF complex. As cyclin B accumulates during interphase, the p34 to which it binds is phosphorylated on Tyr15 within its ATP binding site, thereby suppressing its H1 kinase activity (Solomon et al., 1990; Krek and Nigg, 1991). In *S. pombe*, the inhibitory phosphorylation is carried out by the protein kinases encoded by the *wee1* and *mik1* genes (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren et al., 1991). Analogous protein kinases likely exist in mammalian cells, as application of the protein kinase inhibitors 2-aminopurine or 6-dimethylaminopurine to cells arrested in S phase induces premature chromatin condensation (Schlegel and Pardee, 1986; Schlegel et al., 1990), and a human homolog to *wee1* has recently been cloned (Igarashi et al., 1991).

Thus, unlike what is known of the interphase CDKs, for whose activity cyclin synthesis is rate-limiting, the mitotic CDK must be activated. Activation of the pre-MPF complex containing phosphorylated p34 requires removal of the inhibitory phosphate: in every organism examined, p34 dephosphorylation correlates with H1 kinase activation and the onset of M phase (see Nurse, 1990, for review). In addition, preventing dephosphorylation of p34 in *Xenopus* pre-MPF by addition of p13 blocks activation of H1 kinase activity (Dunphy and Newport, 1989). In fission yeast, mutation of Tyr15 to phenylalanine leads to premature entry into mitosis (Gould and Nurse, 1989), demonstrating that dephosphorylation of Tyr15 is sufficient for mitosis onset. However, p34 from mouse 3T3 cells was not activated by dephosphorylation of tyrosine using a purified human phosphotyrosine phosphatase (Morla et al., 1989). Similarly, microinjection of phosphotyrosine phosphatase 1B into interphase *Xenopus* oocytes did not trigger metaphase onset (Tonks et al., 1990). These results suggest that in animal cells dephosphorylation of Tyr15 is not sufficient to activate p34. In fact, animal cell p34 is also phosphorylated on the adjacent Thr14. Transfection of HeLa cells with p34 cDNA carrying non-phosphorylatable residues in place of Thr14 and Tyr15 rapidly induced premature mitotic events. No effect was seen if Thr14 only was replaced, while replacing only Tyr15 led to delayed, partial effects (Krek and Nigg, 1991), suggesting that in animal cells p34 carries two inhibitory phosphorylations.

A key element in the dephosphorylation of p34 is *cdc25*, a dosage-dependent inducer of mitosis in *S. pombe* and a positive regulator of *cdc2* (Russell and Nurse, 1986), with homologs in *S. cerevisiae* (*MIH1*; Russell et al., 1989), *Drosophila* (*string*; Edgar and O'Farrell, 1989) and human cells (Sadhu et al., 1990). Addition of recombinant *Drosophila* cdc25 to *Xenopus* pre-MPF bound to p13-Sepharose induces dephosphorylation of p34 and the appearance of H1 kinase activity, which is inhibited by addition of soluble p13 (Kumagai and Dunphy, 1991). Although certain temperature-sensitive *S. pombe cdc25* mutants can be rescued by a plasmid-borne human phosphotyrosine phosphatase cDNA (Gould et al., 1990), and although there is limited homology between a portion of cdc25 and a sequence conserved among phosphotyrosine phosphatases (Moreno and Nurse, 1991) that is implicated in substrate recognition (Streuli et al., 1990), initially no cdc25-associated phosphatase activity could be demonstrated; hence, it was concluded that cdc25 most likely activated a phosphatase bound to p13-Sepharose in addition to or perhaps as part of the pre-MPF complex (Kumagai and Dunphy, 1991).

Similar studies were performed adding cdc25 to a more purified substrate complex. p13-Sepharose was used to obtain a pre-MPF complex from oocytes arrested in G_2, and the complex was then purified by chromatography on immobilized cyclin B antibody. Addition of bacterially expressed human cdc25 to the preparation led to dephosphorylation of p34 and activation of H1 kinase activity (Strausfeld et al., 1991). However, when ATP and vanadate were omitted from the purification procedure, the complex was spontaneously activated concomitant with dephosphorylation of the p34 subunit (cf. Labbé et al., 1988, 1989), suggesting the presence of an endogenous phosphatase. SDS-PAGE analysis of the complex indicated the presence of a ~70 kDa polypeptide, even in the purified preparation, though in amounts appearing to be substantially much less than p34 and cyclin B as judged by silver staining (Strausfeld et al., 1991).

Using another approach, in vitro-translated *Xenopus* p34 was bound to cyclin B immobilized on beads, then incubated with a crude interphase extract of *Xenopus* oocytes to phosphorylate p34 (Gautier et al., 1991). Following addition of cdc25 to the system, dephosphorylation of p34 was implied by a shift in its mobility on one-dimensional gels. Concomitant with dephosphorylation was a three-fold increase in H1 kinase activity. As was the case when pre-MPF was used as substrate (see above), activation was inhibited by p13. Although the p34-cyclin B complex was extensively washed following incubation with interphase extract, the possibility could not be completely eliminated that a (cdc25-activatable) phosphatase became associated with the p34-cyclin complex during incubation with interphase extract.

In fact, there is some evidence to suggest that an endogenous p34 phosphatase is tightly associated with the MPF or pre-MPF complex. The 200 kDa complex purified from HeLa cells (Table 1) contained phosphorylated p34 (based on its mobility on SDS-PAGE), yet the complex had H1 kinase activity (Brizuela et al., 1989). This result might be explained if a phosphatase copurified with p34 and cyclin B, and dephosphorylated p34 during the H1 kinase assay. Other observations are also consistent with the presence of a tightly associated p34 phosphatase: p34 is partially dephosphorylated during immunoprecipitation from mitotic 3T3 cells (Morla et al., 1989), while phosphotyrosine is completely removed during immunoprecipitation of p34 from yeast (Potashkin and Beach, 1988).

The p65 component of human MPF, purified to

homogeneity from mitotic cells by immunoaffinity chromatography, is a protein phosphatase with a specific activity against *p*-nitrophenylphosphate (PNPP), which structurally resembles phosphotyrosine, of ~100 μmol P_i/mg per min (Meikrantz et al., 1991a). It dephosphorylated a synthetic polypeptide phosphorylated on tyrosine with a specific activity of ~100 nmol P_i/mg per min, similar to other phosphotyrosine phosphatases purified to homogeneity, and displayed substantial activity against phosphoserine/phosphothreonine as well (Meikrantz et al., 1991a). Addition of purified p65 to purified p34 apoenzyme, phosphorylated on tyrosine (Meikrantz et al., 1991b), stimulated H1 kinase activity thirty-fold, with half-maximal activation occuring at less than 1 mol p65 per mol p34, in the absence of detectable cdc25 (Meikrantz et al., unpublished data). p65 thus appears to be a contender for a phosphatase that activates p34 at mitosis.

However, there is recent evidence that cdc25 is itself a phosphatase. Dunphy and Kumagai (1991) have reported that the C-terminal domain of *Drosophila* cdc25 protein expressed in *Escherichia coli* has phosphatase activity against PNPP. However, the K_m for the reaction was quite large (50 mM), while the specific activity, 2-3 nmol P_i/mg per min (calculated from the data given), is at least several orders of magnitude lower than that expected for a purified phosphatase. These values could be the result of non-specific catalysis on the refolded cdc25 surface rather than true enzymatic hydrolysis. Since *O*-phosphate hydrolysis has a negative free energy at alkaline pH, it is possible that at the pH of 8.2 used in the PNPP assays cdc25 might be promoting general base catalysis of the phosphate ester by providing a suitable electron donor, consistent with the great reduction in hydrolysis at neutral pH reported by the authors. Significantly, hydrolysis was not affected by p13, indicating that the reaction was likely distinct from that catalyzed by cdc25 in more complex systems (see above).

The cdc25 fragment was also found to dephosphorylate Tyr15 in a p34-derived peptide as well as tyrosine-phosphorylated angiotensin II (Dunphy and Kumagai, 1991). Only 1.2% of added substrate was hydrolyzed after 3 h at 37°C, however, representing a six-fold increase above background. Although hydrolysis did not occur with a cdc25 fragment in which a conserved cysteine was mutated, the cysteine could be the electron donor required for general base-catalyzed hydrolysis. While the activity was inhibited by vanadate, which inhibits phosphotyrosine phosphatases, vanadate is known to exert many of its cytotoxic effects by specific ablation of cysteine residues (Friedman, 1973). Alternatively, since both ortho- and meta-vanadate are reduced to the 4+ oxidation state in aqueous solution (Cantley and Aisen, 1979), base catalysis might be quenched by vanadate acting as an electron sink (irrespective of any specific effect on cysteine); in fact, background hydrolysis of the labeled peptide was lower in the presence of cdc25 and vanadate than in the absence of cdc25 (Dunphy and Kumagai, 1991).

In contrast to these results, Gautier et al. (1991) find that recombinant cdc25 is not able to hydrolyze either PNPP or phosphorylated Tyr15 in a p34-derived peptide. However, this recombinant cdc25 did promote dephosphorylation of a more complex substrate. In vitro-translated *Xenopus* p34 was phosphorylated with ^{32}P on Tyr15 by purified src. Addition of cdc25 to this substrate resulted in dephosphorylation of p34 as judged by loss of radioactivity from p34 on autoradiographs of one-dimensional gels and by two-dimensional peptide maps. In this case, dephosphorylation was unaffected by p13.

Perspectives: the Rapkine cycle and MPF

Some forty years prior to Hartwell's characterization of yeast *cdc* mutants, Rapkine (1931) reported a sudden decrease in the concentration of soluble sulfhydryl groups coincident with the onset of mitosis in sea urchin embryos. This decrease could be blocked by the addition of thiol reagents, which also prevented the cells from entering mitosis, leading to the proposal that oxidation of protein sulfhydryls was the driving force behind the cell cycle (see discussions by Mazia, 1955, 1958; Luca and Ruderman, 1989; Meikrantz et al., 1990).

Although linking of polypeptides by disulfides is not particularly common within the cell, there are notable examples, such as the regulatory subunit homodimer of cAMP-dependent protein kinase and the homodimeric cGMP-dependent protein kinase, the latter being notoriously difficult to maintain in the reduced state (Flockhart and Corbin, 1982). In addition, a number of other proteins are known to be regulated via their oxidation/reduction status, including the murine protein tyrosine kinase ltk (Bauskin et al., 1991) and cGMP-dependent protein kinase (Landgraf et al., 1991). And in accord with the spirit of Rapkine's hypothesis, growth arrest by TGFβ is mediated through the reducing action of thioredoxin (Deiss and Kimchi, 1991), while the growth-associated DNA-binding activity of the transcription factors AP-1 and NF-κB is mediated by oxidation (Abate et al., 1990; Schreck et al., 1991; Staal et al., 1990).

Disulfide bonds in the p65 dimer seem to be necessary for expression of its phosphatase activity (Meikrantz et al., 1991a), while formation of the dimer correlates with its ability to bind p34 and cyclin B (Meikrantz et al., 1990). Conversely, in order for cdc25 to catalyze the pre-MPF to MPF conversion, mild reducing conditions are required, while alkylation with *N*-ethylmaleimide blocks its catalytic activity (Dunphy and Kumagai, 1991). This combination of proteins with differing oxidation potentials suggests a mechanism by which the Rapkine cycle might be integrated into the pathway of MPF activation: the free cdc25 thiol might donate the electron required for p65 dimer formation. Alternatively, p65, activated by oxidation, and cdc25 may simply act redundantly to dephosphorylate p34, just as wee1 and mik1 act redundantly in its phosphorylation.

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