Essential Roles for Calcium and Calmodulin in G2/M Progression in *Aspergillus nidulans*

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Abstract. *nimT* encodes a protein in *Aspergillus nidulans* that is required for tyrosine dephosphorylation of $p34^{cdc2}$ and has a strong homology to cdc25-type proteins. Conditional mutation of *nim*T (*nim*T23 mutation) arrests cells in G2 at the restrictive temperature. After release of the temperature-sensitive *nim*T23 block, $p34^{cdc2}$ undergoes tyrosine dephosphorylation and we showed that as cells entered mitosis, a rapid increase in calmodulin was observed. The increase in calmodulin and progression into mitosis were prevented by reducing extracellular Ca^{2+} levels to 2 nM. The calmodulin gene of a *nim*T23-containing strain was replaced with a hybrid gene in which calmodulin transcription was regulated by the *alc*A promoter (AlcCaM/T23). This allowed experimental manipulation of the level of intracellular calmodulin by the carbon source in the medium. When either extracellular Ca^{2+} or intracellular calmodulin levels were reduced at the *nim*T23 G2 arrest point, $p34^{cdc2}$ remained tyrosine phosphorylated but the mitotic NIMA kinase encoded by *nim*A was not activated. Release of the temperature sensitive *nim*T23 arrest when either extracellular Ca^{2+} or calmodulin concentrations were low blocked tyrosine dephosphorylation of $p34^{cdc2}$, activation of NIMA and progression of cells into mitosis. However, reduced levels of either Ca^{2+} or calmodulin had no effect on the increase in histone H1 kinase activity associated with p13 beads or the degree of phosphorylation of the majority of MPM-2–reacting proteins following release of the *nim*T23 mutation. These results demonstrate that both Ca^{2+} and calmodulin are important for progression into mitosis from the *nim*T23 arrest point in a pathway involving activation of both NIMA and $p34^{cdc2}$ protein kinases.

CALCIUM has been shown to be critical for G2/M progression in marine eggs (for review see Whitaker and Patel, 1990). For example, transient increases in intracellular free Ca^{2+} are associated with nuclear envelope breakdown in sea urchin eggs (Poenie et al., 1985) and microinjection of the Ca^{2+} chelators EGTA or BAPTA blocks breakdown of the nuclear envelope (Steinhardt and Alderton, 1988; Twigg et al., 1988). Causing a premature increase in intracellular Ca^{2+} by microinjecting either Ca^{2+} or IP_3 induces a rapid and precocious entry into mitosis (Twigg et al., 1988). These results indicate that the Ca^{2+} transients are both necessary and sufficient for the initiation of mitosis in sea urchin eggs. Similarly, in clam eggs, a requirement for Ca^{2+} has been shown for the initiation of germinal vesicle breakdown and meiosis immediately following fertilization which triggers influx of extracellular Ca^{2+} (Guerrier et al., 1981; Dube et al., 1987; Bloom et al., 1988). Considerable evidence is also available that points to an importance for Ca^{2+} and calmodulin in the G2 to mitosis transition of mammalian cells (for review see Means et al., 1991). Both Ca^{2+} and calmodulin are concentrated in the centrosomal region of the mitotic spindle (Welsh et al., 1978; 1979; Wolniak et al., 1980). Transient increases in intracellular Ca^{2+} have been shown to be associated with nuclear envelope breakdown and chromatin condensation (Keith et al., 1985; Kao et al., 1990). Artificially elevated cytosolic free Ca^{2+} concentrations were shown to induce premature breakdown of the nuclear envelope which could be blocked by a decrease in intracellular Ca^{2+} levels (Kao et al., 1990). Reduction of functional calmodulin accomplished by either exposure to calmodulin antagonists or expression of calmodulin antisense RNA blocks progression into mitosis (Rasmussen and Means, 1989; Sasaki and Hidaka, 1982; Chafouleas et al., 1982), indicating the involvement of calmodulin. However, because of the pleotypic effects of Ca^{2+} and calmodulin, it could be argued that the cell effects are of a general rather than a specific nature.

To address the nature of the role for calmodulin in cell cycle progression, we created a strain of *Aspergillus nidulans* in which calmodulin expression is conditional and demonstrated that manipulation of calmodulin levels allowed induction of a reversible G2 arrest of the nuclear division cycle (Lu et al., 1992). Threshold levels of intracellular calmodulin

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were found to be required for release from the block and subsequent entry of the cells into mitosis (Lu et al., 1992; Rasmussen et al., 1992). Overexpression of calmodulin both increased the rate of growth and decreased the requirement for optimal extracellular Ca^{2+} by a factor of 10. Whereas it is formally possible that these requirements are not linked and that the essential function of calmodulin does not require Ca^{2+} binding as is the case in *Saccharomyces cerevisiae* (Geiser et al., 1991), these observations support the notion that Ca^{2+} and calmodulin are required for cells to progress from G2 into mitosis in *A. nidulans*. However, nothing is known about whether Ca^{2+}/calmodulin affects some selective protein targets at this transition of cell cycle progression.

Genes required for G2/M progression encode both protein kinases and phosphatases. The protein kinase ($p34^{cdc2}$) encoded by the *cdc*2 gene of *S. pombe* and its homologs in other species is the most widely studied of the mitotic protein kinases and has been demonstrated to be functionally conserved (Beach et al., 1982; Lee and Nurse, 1987; Murray and Kirschner, 1989; Nurse, 1990). The $p34^{cdc2}$ protein kinase is the catalytic subunit of maturation promotion factor (MPF),[1] a multi-protein complex that includes $p34^{cdc2}$ and cyclin B, and is thought to regulate mitosis and meiosis in all eukaryotes (Arion et al., 1988; Gautier et al., 1988; Labbe et al., 1989). Activity of the $p34^{cdc2}$ protein kinase has been shown to be modulated posttranscriptionally by tyrosine and threonine phosphorylation/dephosphorylation and interaction with cyclin proteins. The phosphorylation of $p34^{cdc2}$ on a threonine residue (amino acid [aa] 167 in fission yeast, or aa 161 in *Xenopus*) seems important for binding to mitotic cyclin (Gould et al., 1991; Norbury et al., 1991) and for mitotic progression (Gould et al., 1991; Solomon et al., 1992). During interphase, cyclin binding targets $p34^{cdc2}$ for tyrosine phosphorylation (aa 15 in fission yeast; Gould and Nurse, 1989; Meijer et al., 1991). Two protein kinases, WEE1 and MIK1, have been shown to be responsible for $p34^{cdc2}$ tyrosine phosphorylation, resulting in an inactive $p34^{cdc2}$ (Featherstone and Russell, 1991; Lundgren et al., 1991). During the G2/M transition, the protein encoded by the *cdc*25 gene of *S. pombe*, and its homolog in other systems, is activated. The active cdc25 protein is a tyrosine phosphatase which specifically removes tyrosine phosphate from $p34^{cdc2}$, thereby activating the protein kinase (Gould and Nurse, 1989; Morla et al., 1989; Dunphy and Newport, 1989; Solomon et al., 1990; Dunphy and Kumagai, 1991; Gautier et al., 1991).

A homolog of *cdc*25 in *A. nidulans* has recently been identified as the product of the *nim*T gene (Osmani et al., 1991*a*; O'Connell et al., 1992). Strains containing a temperature sensitive mutation of $nimT^{cdc25}$ (*nim*T23) are arrested in G2 at the restrictive temperature with $p34^{cdc2}$ tyrosine phosphorylated. Upon release from the block, $p34^{cdc2}$ kinase is tyrosine dephosphorylated and activated, resulting in entry of cells into mitosis. However, whereas activation of $p34^{cdc2}$ kinase is required, it is not sufficient to trigger mitosis in *A. nidulans* if the NIMA protein kinase encoded by *nim*A is not activated (Osmani et al., 1991*a*). The NIMA kinase is a cell cycle–dependent β-casein kinase that exhibits elevated activity during late G2 and M (Osmani et al., 1991*b*). Temperature sensitive mutations of *nim*A cause a G2 arrest at the restrictive temperature. During the block, $p34^{cdc2}$ kinase is tyrosine dephosphorylated and fully activated. Upon returning to the permissive temperature, the arrested cells rapidly and synchronously enter mitosis, demonstrating that the NIMA kinase is also required to enter mitosis. These results indicate that activation of both $p34^{cdc2}$ and NIMA kinases are mandatory for mitosis in *A. nidulans* (Osmani et al., 1991*a*).

Since mitotic progression requires the activities of the $p34^{cdc2}$ and NIMA protein kinases in *A. nidulans*, and this cell cycle transition also requires Ca^{2+} and calmodulin, we have questioned whether extracellular Ca^{2+} and calmodulin would affect activation of either one or both of these mitotic kinases. Our results indicate that Ca^{2+} and calmodulin are not required for tyrosine phosphorylation of $p34^{cdc2}$ at the *nim*T23 G2 arrest point, nor required for the increase in histone H1 kinase activity associated with p13 beads or most protein phosphorylation detected by the MPM-2 mAb after release of the temperature sensitive *nim*T23 mutation. However, both Ca^{2+} and calmodulin are required for entry of cells into mitosis, activation of the NIMA kinase, and tyrosine dephosphorylation of $p34^{cdc2}$.

Materials and Methods

A. nidulans *Strains and General Techniques*

Strains used in this study were R153 (*w*A3; *pyro*A4), SO7 (*nim*A5) (*nim*A5;*w*A2), SO53 (*nim*T23) (*nim*T23; *w*A2), PL0 (AlcCaM) (*alc*:calmodulin; *w*A2; *pyro*A4), and PL7 (AlcCaM/T23) (*alc*:calmodulin; *nim*T23; *w*A2; *bi*A1; *paba*A1). The AlcCaM/T23 strain was created from SO26 (*nim*T23; *pyr*G89; *w*A2; *bi*A1; *paba*A1) by transformation with plasmid pAL-CaMKP containing a pAL3 vector and a copy of the calmodulin gene with a 3′ deletion driven by the *alc*A promoter, as described previously (Lu et al., 1992; Lu and Means, 1993). Growth and fluorescence microscopy of *A. nidulans* were as described previously (Rasmussen et al., 1990; Lu et al., 1992), as were cell cycle blocks (Osmani et al., 1991*a*).

Measurement of Ca^{2+} and Calmodulin Concentrations

Intracellular calmodulin concentrations of *A. nidulans* were assayed by the radioimmunoassay developed by Chafouleas et al. (1979) and modified by Rasmussen et al. (1990), while calcium concentrations in the chemically defined growth media were determined by atomic absorption spectrometry, as described previously (Lu et al., 1992).

NIMA Protein Kinase and Histone H1 Kinase Assays

NIMA kinase assays were carried out using β-casein as a substrate after immunoprecipitation with NIMA specific antibodies, while the H1 kinase was assayed in p13 precipitates using histone H1 as a substrate, as described by Osmani et al. (1991*a*,*b*).

Immunoblot Analysis

Preparation of cell extracts and p13 affinity purification of $p34^{cdc2}$ were performed as described (Osmani et al., 1991*a*). For detecting MPM-2 antigens, 200 μg of soluble cell extracts were directly solubilized in SDS buffer. The proteins were separated on 10% acrylamide gels in the presence of SDS and transferred to Immobilon-P membranes. The filters were blocked for 1 h in TBS, pH 7.4, containing 5% bovine albumin. Subsequently, filters were incubated for 2 h at room temperature and either anti-phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY), anti-PSTAIR antibody, anti-$p34^{cdc2}$, or MPM-2 antibody, followed by five washes with TBS:Tween 20 (0.05%). To determine which anti-PSTAIR antibody-reacting proteins contained Tyr phosphate, filters containing separated p13 bind-

1. *Abbreviations used in this paper*: aa, amino acid; MPF, maturation promoting factor.

ing proteins (three- to fivefold more than usual) were probed sequentially with the anti-phosphotyrosine antibody and then with anti-PSTAIR antibody. To detect phosphotyrosine, the filters were incubated for 1 h in TBS containing [^{125}I]-protein A (ICN Radiochemicals, Irvine, CA), followed by five washes of 5 min each and then subjected to autoradiography. For using anti-PSTAIR or MPM-2 antibodies, the filters were incubated with alkaline phosphatase-conjugated secondary antibodies (Promega Biotec, Madison, WI) and binding of the secondary antibodies was visualized as described previously (Kuang et al., 1989). Alternatively, the filters were incubated with HRP conjugated anti-mouse IgG, and a ECL detection system was used according to the manufacturer's procedure (Amersham Corp., Arlington Heights, IL). To semi-quantify the MPM-2 stainings, films were scanned by LKB Ultroscan.

Phosphatase Treatments

To examine the effect of phosphatases on the mobility of PSTAIR-containing proteins, the cell extracts were incubated either with p13 beads for 2 h and then washed three times with HK buffer and five times with potato acid phosphatase buffer consisting of 50 mM Pipes, pH 6.0, 0.1% β-mercaptoethanol, 0.1 mM PMSF, 0.1 mM benzamidine, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml leupeptin (Pondaven et al., 1990), or calf alkaline phosphatase buffer consisting of 50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine, 0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml leupeptin according to the manufacturer's procedure (Boehringer Mannheim Biochemicals, Indianapolis, IN). The p13-binding proteins were incubated for 10 min at 30°C with different amounts of potato acid phosphatase (Sigma Immunochemicals, St. Louis, MO) or calf alkaline phosphatase (Boehringer Mannheim Biochemicals), respectively, and then solubilized in SDS buffer for Western analysis.

Results

Increase in Calmodulin Levels as Cells Enter Mitosis

We previously reported an increase in the level of calmodulin late in the cell cycle of a strain of *A. nidulans* (Rasmussen et al., 1990). This was discovered by monitoring calmodulin levels in cells entering the cell cycle from dormant spores (conidia). To more precisely examine the relationship between changes in the calmodulin level and entry into mitosis, we have used a *nim*T23 temperature-sensitive mutation in the *nim*T^{cdc25} gene to first arrest cells in G2 and then, by a shift to the permissive temperature, allow a synchronous nuclear division to take place. Incubation of *nim*T23 cells in normal medium at the restrictive temperature for 2.5 h results in arrest of >90% of the cells in G2 (Osmani et al., 1991*a*). Therefore, a strain containing the *nim*T23 mutation was grown to early log phase at the permissive temperature and the culture was then shifted to the restrictive temperature for 3 h to arrest cells in G2. Before and after returning the culture to the permissive temperature, we monitored the chromosome mitotic index and the levels of calmodulin, calmodulin mRNA, and histone H3 mRNA (Fig. 1 *top*, and data not shown). As a control, the nontemperature-sensitive R153 strain was used; no significant changes in the parameters tested occurred in this strain during the temperature shifts (Fig. 1 *bottom*). However, when the *nim*T23-containing cells were released from the G2 block, changes in calmodulin levels occurred in concert with changes in the chromosome mitotic index (Fib. 1 *top*). This rapid increase in calmodulin during the onset of mitosis was not preceded by an increase in calmodulin mRNA (data not shown). Similar results to those described above using the *nim*T23 mutation were also obtained using another temperature-sensitive strain, *nim*A5, that contains a temperature-sensitive mutation of the *nim*A gene and is also reversibly arrested in G2 (data not shown). These data indicate that progression into mitosis in *A. nidulans* is associated with a rapid increase in the level of calmodulin that appears to be regulated posttranscriptionally.

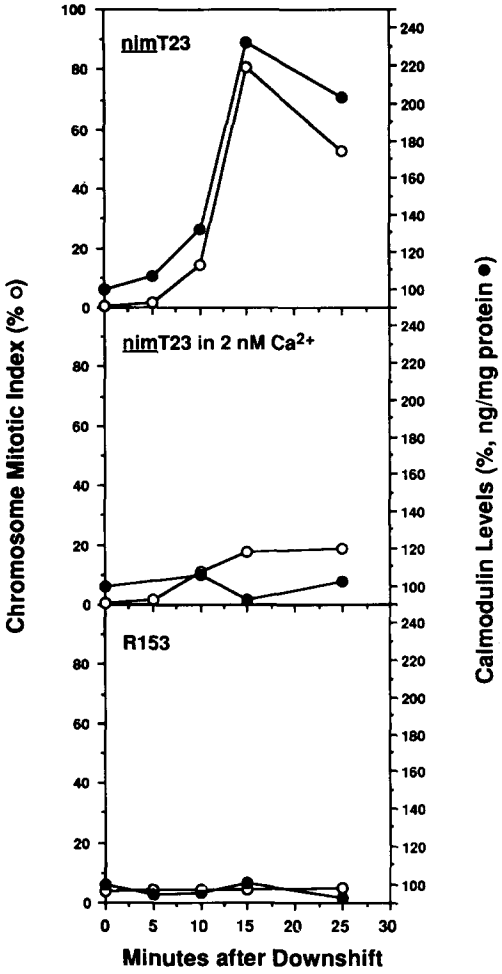

Figure 1. Changes in calmodulin levels as cells enter mitosis. Conidia from the temperature-sensitive *nim*T23 (*top* and *middle*) or nontemperature-sensitive R153 strain (*bottom*) were grown until early log phase at 25°C and then shifted to 42°C for 3 h (time *0*). For low Ca^{2+} culture (*middle*), EGTA was added to reduce extracellular Ca^{2+} levels to 2 nM for 2.5 h during incubation at the restrictive temperature. The cultures were then placed in ice water to decrease the temperature to 25°C and incubated at this temperature for the times indicated. Chromosome mitotic indexes were determined by counting the percentage of cells without defined nucleoli but with shape changed and condensed nuclei, after DAPI staining, as described (Osmani et al., 1991*a*) and calmodulin levels were determined by radioimmunoassay, as described (Rasmussen et al., 1990).

Requirement of Ca^{2+} for the Calmodulin Increase and Progression into Mitosis

Since Ca^{2+} has been shown to be required for protein translation (for review see Brostrom and Brostrom, 1990), we examined the effect of extracellular Ca^{2+} on the increase in calmodulin after release of the G2 arrest, as shown in Fig. 1 (*middle*). The *nim*T23 cells were grown to early log phase at the permissive temperature and then shifted to the restrictive temperature. After 1 h at the restrictive temperature, the

extracellular Ca^{2+} concentration was reduced to 2 nM by the addition of EGTA for 2 h and the cultures were then returned to the permissive temperature, followed by measuring the chromosome mitotic index and the levels of calmodulin, as described above. There was no significant change in calmodulin levels in low Ca^{2+} conditions and neither could the cells enter mitosis. These results suggest that extracellular Ca^{2+} is required for the calmodulin increase as well as entry into mitosis following release of the *nim*T23 mutation.

To further examine the requirement of extracellular Ca^{2+} for progression of the *nim*T23 arrested cells into G2, we evaluated progression into mitosis as a function of time that the cells were exposed to low extracellular Ca^{2+}. As shown in Fig. 2 *A*, EGTA was added at different times during which the cells were held at the restrictive temperature and the mitotic index was determined at various times after returning the cells to the permissive temperature. To block cells that were released from G2 in mitosis making them easier to monitor the G2/M transition, the microtubule-depolymerizing drug benomyl was added at a concentration of 5 μg/ml 10 min immediately before the temperature downshift, as described previously (Osmani et al., 1991*a*). The control cells (without adding EGTA) readily entered mitosis from the G2 arrest and so did those to which EGTA was added 10 min before the temperature shift (Fig. 2 *B*). However, when cells were incubated in the presence of EGTA for 2.5, 2 or 1 h at the restrictive temperature, and then released from the G2 block, the majority of cells did not enter mitosis (Fig. 2 *B*). Fig. 2 *B* also shows that increasing extracellular Ca^{2+} to 1 mM by adding $CaCl_2$ just 10 min before the temperature downshift allowed cells to enter mitosis as rapidly as control cells, indicating that under the conditions used, the effect of reduced levels of extracellular Ca^{2+} is fully reversible. These results suggest that reducing extracellular Ca^{2+} may decrease intracellular Ca^{2+} levels, thereby blocking progression into mitosis.

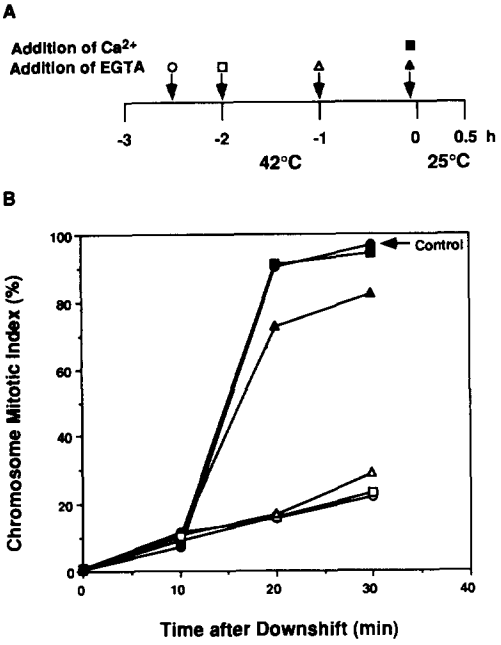

Figure 2. Effect of reduced extracellular Ca^{2+} levels on entry into mitosis from the *nim*T23 arrest point. The *nim*T23 strain was grown to early log phase at 25°C and then shifted to 42°C for 3 h. EGTA and/or Ca^{2+} were added at different times during the restrictive temperature as indicated in *A* to bring the extracellular free Ca^{2+} to 2 nM or 1 mM, while water was added as control. The cultures were shifted to 25°C in the presence of 5 μg/ml benomyl (time *0*) and then incubated at this temperature for the times indicated in *B*. Samples were removed to count the percentage of cells with mitotic figures. (●) control; (○, □, △, ▲) EGTA was added at 2.5, 2, 1, and 0.17 h, respectively, before the downshift; ■, EGTA was added at 2.5 h but $CaCl_2$ was added at 0.17 h before the downshift to bring the extracellular free Ca^{2+} to 1 mM.

Effect of Reduced Intracellular Calmodulin Levels on Progression into Mitosis from the nim*T23 Arrest Point*

An increase in calmodulin levels has been implicated to be required for cell cycle progression in animal cells (for review see Means et al., 1991). To examine a potential role for the calmodulin increase during mitosis in *A. nidulans*, we introduced a mutation conditional for calmodulin expression into the genetic background of the *nim*T23 strain, generating an AlcCaM/T23 strain, as described previously (Lu et al., 1992). The AlcCaM/T23 strain, like the AlcCaM strain generated in the wild type genetic background (Lu et al., 1992), was found to be dependent on the activity of the *alc*A promoter for growth. When grown in glycerol (noninduced/nonrepressed for the *alc*A promoter), both AlcCaM/T23 and AlcCaM strains were able to grow normally but when placed in repressing glucose and/or acetate media shutting off calmodulin expression, cells did not grow (data not shown). There was no detectable difference between the AlcCaM/T23 and AlcCaM strains when grown at the permissive temperature, but at the restrictive temperature, the AlcCaM/T23 strain could not grow even in glycerol-containing medium, while the AlcCaM strain did grow in the same medium (data not shown). These results indicate that the AlcCaM/T23 strain contains an *alc*A promoter-regulated calmodulin gene as well as a temperature-sensitive *nim*T23 mutation.

With the AlcCaM/T23 strain, it is possible to examine the requirement of calmodulin for entry into mitosis by arresting cells in G2 with the *nim*T23 mutation in the presence of different levels of calmodulin and then examining the ability of these cells to enter mitosis after release of the temperature-sensitive mutation. When spores from AlcCaM/T23 strain were germinated in repressing medium at the permissive temperature, most cells were also able to undergo one nuclear division and then arrested in G2 (data not shown), as was previously shown to be the case for the AlcCaM strain (Lu et al., 1992). To synchronize the majority of cells in G2 of the first nuclear division cycle, spores were first germinated in either repressing or inducing medium for 4.5 h and then shifted to the restrictive temperature for an additional 4.5 h. This protocol resulted in the arrest of over 95% of the cells in G2 with one nucleus per cell regardless of which medium was used. Therefore the G2 arrest was due to activation of the temperature-sensitive *nim*T23 mutation. However, as shown in Fig. 3 *A*, calmodulin levels were influenced by the carbon source present in the culture media and decreased in repressing medium but increased in inducing medium to ~5 or 300% of the calmodulin level present in control *nim*T23 cells, respectively. These cells containing different calmodu-

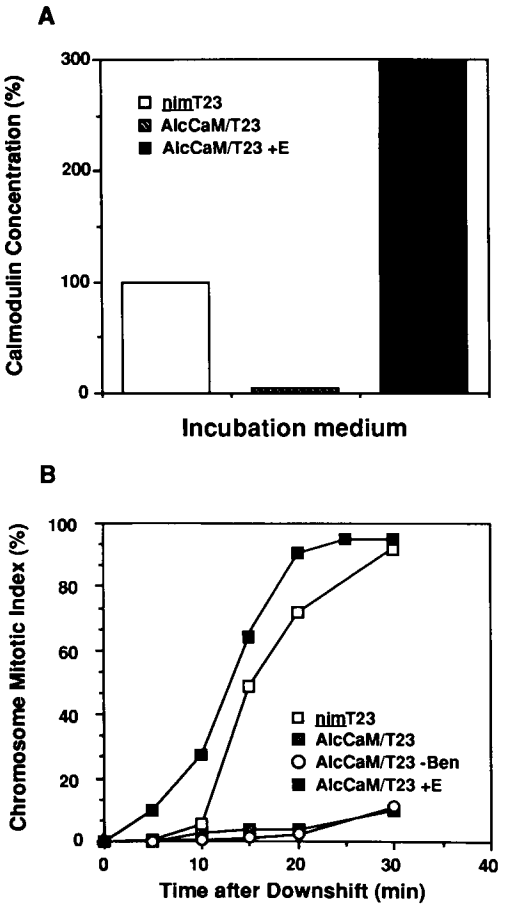

Figure 3. Effect of intracellular calmodulin levels on entry into mitosis from the *nim*T23 arrest point. The *nim*T23 and AlcCaM/T23 conidia were grown in repressing medium or inducing medium (*E*) at 32°C for 4.5 h and then at 42°C for 4.5 h (time *0*). Samples were removed to measure calmodulin levels by radioimmunoassay (*A*) and, except for one culture of AlcCaM/T23 cells (-Ben), benomyl was added to a final concentration of 5 μg/ml for 10 min before downshifting to 25°C. After downshift for the times as indicated, samples were removed to count the percentage of cells with mitotic figures (*B*), as described in Fig. 1. (□, □) *nim*T23 cells in repressing medium; (□, ▨), AlcCaM/T23 cells in repressing medium; (□, ■) AlcCaM/T23 cells in inducing medium; (○) AlcCaM/T23 cells in repressing medium in the absence of benomyl.

lin concentrations were then returned to the permissive temperature in the presence or absence of benomyl. In low calmodulin conditions, the AlcCaM/T23 strain was severely impaired in its ability to enter mitosis upon release from the G2 arrest point when compared to the same cells grown in inducing medium or to control *nim*T23 cells (Fig. 3 *B*). At 30 min following release from G_2 arrest, over 90% of the *nim*T23 cells or AlcCaM/T23 cells grown in inducing medium had entered mitosis. In contrast, only ~15% of the AlcCaM/T23 cells entered mitosis after release from the G_2 block when grown in repressing media. Fig. 3 *B* also reveals that the inhibitory effect of reduced calmodulin levels on entry into mitosis was seen both in the presence and the absence of benomyl, indicating that the effects of reduced calmodulin levels are not due to the presence of benomyl. A similar effect of reduced levels of calmodulin was also observed in AlcCaM/T23 cells that had been grown to early log phase (data not shown). These results demonstrate that threshold levels of intracellular calmodulin are required for the cells to enter mitosis upon release from the *nim*T23-arrest point.

Effect of Reduced Calmodulin Levels on NIMA Protein Kinase Activity and Tyrosine Phosphorylation/Dephosphorylation of $p34^{cdc2}$

Activation of both NIMA and $p34^{cdc2}$ has been shown to be essential for the initiation of mitosis in *A. nidulans* (Osmani et al., 1991*a*). To identify potential targets that might be affected by reducing calmodulin levels, we examined NIMA activity and tyrosine phosphorylation of $p34^{cdc2}$ in the presence of different calmodulin levels. Conidia from the AlcCaM/T23 and control *nim*T23 strains were germinated in repressing or inducing medium at the permissive temperature and then placed at the restrictive temperature to arrest cells in G2, followed by release into mitosis, as described above. The cells before (as indicated in figures as 0) and after release into benomyl for 30 min (30) were harvested for assay of NIMA kinase activity, $p34^{cdc2}$ tyrosine phosphorylation, H1 kinase activity, and phosphorylation of the MPM-2-reacting proteins, as described in Materials and Methods. As shown previously (Osmani et al., 1991*a*), when the *nim*T23 cells were arrested in G2, NIMA kinase was fully active, with an activity similar to that in mitotic cells (Fig. 4 *A*). However, when calmodulin in the AlcCaM/T23 strain was reduced to ~5% of the control level in repressing medium (Fig. 3 *A*), NIMA was not activated at the *nim*T23 arrest point, having an activity similar to that present in G1 cells, and only increased slightly after release from the arrest (Fig. 4 *A*). In contrast, if similarly treated cells were incubated in inducing medium and contained increased calmodulin levels, NIMA activity was high at the G2 arrest point as is the case in the control *nim*T23 cells (data not shown). These results indicate that the increase in NIMA kinase activity associated with the arrest of cells in G2 and mitosis is not observed when cells contain low levels of calmodulin.

As shown in the upper panel of Fig. 4 *B*, when the *nim*T23 cells were arrested in G2, $p34^{cdc2}$ was found to be tyrosine phosphorylated and the tyrosine phosphate disappeared following release from the *nim*T23 mutation, consistent with previously reported data (Osmani et al., 1991*a*). In the presence of reduced levels of calmodulin, similar levels of tyrosine phosphate on $p34^{cdc2}$ were observed at the G2 arrest point (Fig. 4 *B*, *top*). However, $p34^{cdc2}$ remained tyrosine phosphorylated after release of the *nim*T23 temperature-sensitive mutation (Fig. 4 *B*, *top*). Immunoblot analysis using the anti-PSTAIR antibody revealed that each sample contained equivalent amounts of $p34^{cdc2}$ (Fig. 4 *B*, *bottom*), indicating that the observed differences in tyrosine phosphate on $p34^{cdc2}$ is not due to different amounts of proteins precipitated by the p13 beads. These results show that low calmodulin conditions prevent tyrosine dephosphorylation of $p34^{cdc2}$.

Effects of Reduced Ca^{2+} Levels on NIMA Kinase Activity and Tyrosine Phosphorylation/Dephosphorylation of $p34^{cdc2}$

Since reduced levels of extracellular Ca^{2+} prevented the cal-

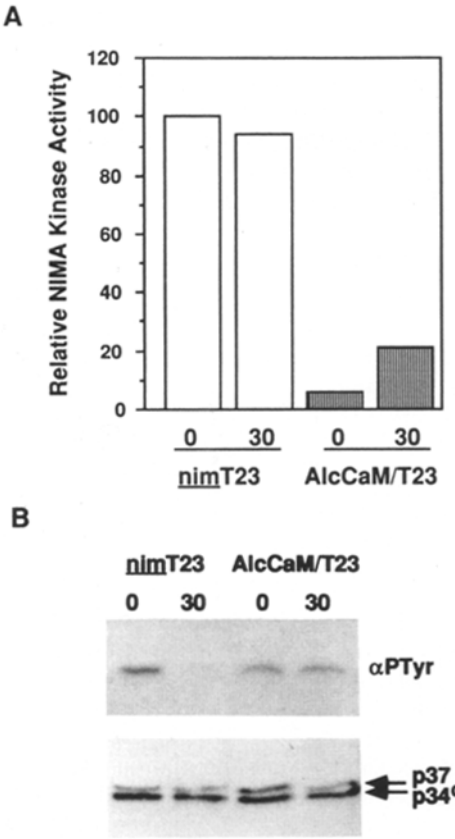

Figure 4. Effect of reduced calmodulin levels on NIMA kinase activity and tyrosine phosphorylation/dephosphorylation of $p34^{cdc2}$. (*A*) Effect on NIMA activity. The *nim*T23 and AlcCaM/T23 cells were arrested in G2 of the first cell cycle in repressing medium at 42°C (*0*) and then at 25°C for 30 min (*30*) in the presence of benomyl as described in Fig. 3. Samples were assayed for NIMA protein kinase activity using β-casein as a substrate, as described (Osmani et al., 1991*a*). Phosphorylated β-casein is shown as the relative activities determined by counting the β-casein bands excised from the dried gel (100% = the maximal activity observed in control *nim*T23-arrested cells). (□) *nim*T23 block and release; (▥) AlcCam/T23 block and release. (*B*) Effect on tyrosine phosphorylation/dephosphorylation. Aliquot of the samples same as *A* were ground in HK buffer and used to isolate the p13 binding proteins using p13 beads. Half of the p13-binding proteins were probed with the monoclonal anti-phosphotyrosine antibody and the other half was probed with the anti-PSTAIR antibody, after Western blotting.

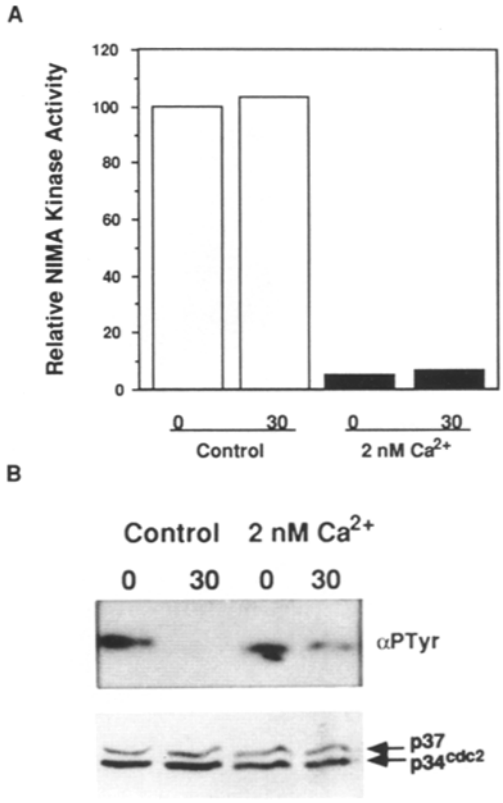

Figure 5. Effect of reduced extracellular Ca^{2+} levels on NIMA kinase activity and tyrosine phosphorylation/dephosphorylation of $p34^{cdc2}$. (*A*) Effect on NIMA activity. The *nim*T23 cells were arrested in G2 at 42°C (*0*) in media containing different Ca^{2+} concentrations as described in Fig. 1 and then at 25°C for 30 min (*30*) in the presence of benomyl. Cells were harvested and assayed for NIMA protein kinase activity (100% = the maximal activity observed in control *nim*T23-arrested cells). (□) *nim*T23 block and release in normal Ca^{2+}; (■) AlcCaM/T23 block and release in low Ca^{2+}. (*B*) Effect on tyrosine phosphorylation/dephosphorylation. The same samples as *A* were used to isolate the p13 binding proteins, followed by Western analysis using the anti-phosphotyrosine or anti-PSTAIR antibody.

modulin increase at mitosis as well as entry of the *nim*T23 cells into mitosis, we also examined the effect of reduced extracellular Ca^{2+} levels on NIMA kinase activity and tyrosine phosphorylation of $p34^{cdc2}$, as shown in Fig. 5. The *nim*T23 cells were arrested in G2 at the restrictive temperature in the presence of 2 nM or 1 mM extracellular Ca^{2+} for 2 h and then released into mitosis in the presence of benomyl, as described earlier. The samples were collected at the G2 arrest point (0) and 30 min after the temperature downshift (30). The increase in NIMA activity usually observed when *nim*T23 cells are arrested in G2 or at mitosis was not observed if extracellular Ca^{2+} levels were reduced to 2 nM (Fig. 5 *A*). If extracellular Ca^{2+} concentration was increased to 1 mM, normal activation of NIMA activity was then observed (data not shown). Furthermore, while reduced extracellular Ca^{2+} concentrations did not affect tyrosine phosphorylation of $p34^{cdc2}$ at the *nim*T23 arrest point, they substantially inhibited removal of tyrosine phosphate from $p34^{cdc2}$ following release of the *nim*T23 mutation, although this block was not as effective as lowering intracellular calmodulin levels, (Fig. 5 *B, top*). This may be due to the presence of some intracellular Ca^{2+} even in the presence of only 2 nM extracellular Ca^{2+}. Since the protein levels of $p34^{cdc2}$ were quite similar among lanes as indicated in the bottom panel of Fig. 5 *B*, these results indicate that in the presence of low Ca^{2+} levels, both NIMA activity and tyrosine dephosphorylation of $p34^{cdc2}$ were inhibited.

Effects of Reduced Levels of Calmodulin or Ca^{2+} on the Histone H1 Kinase Activity Associated with p13 Beads following Release of the nim*T23 Arrest*

As shown in Fig. 4 and 5, we also detected another protein with M_r 37 kD (p37) in p13 precipitates which was recog-

nized by the anti-PSTAIR antibody, but not by the anti-phosphotyrosine antibody. The p37 protein could be a $p34^{cdc2}$-related protein, or a posttranslationally modified form of $p34^{cdc2}$. The most common posttranslational modification of $p34^{cdc2}$ is protein phosphorylation and phosphorylation can impede the mobility of the protein on SDS-containing polyacrylamide gels (Pondaven et al., 1990). To examine the possibility that p37 could be a phosphorylated form of $p34^{cdc2}$, p13 precipitates were pretreated with two different phosphatases: potato acid phosphatase and calf alkaline phosphatase before being subjected to immunoblot analysis. Neither phosphatase treatment using 1 U potato acid phosphatase or 10 U calf alkaline phosphatase changed the ratio of p37 to $p34^{cdc2}$ detected with anti-PSTAIR (Fig. 6 *A*). However, even at 10-fold lower concentrations, both phosphatases did completely remove tyrosine phosphate from $p34^{cdc2}$, with potato acid phosphatase being about 10-fold more potent than calf alkaline phosphatase (Fig. 6 *B*). These results suggest that p37 protein may be a distinct gene product (e.g., a homolog of $p37^{cdk3}$; Meyerson et al., 1992) although we cannot rule out a form of $p34^{cdc2}$ modified in some way we cannot detect.

Since p13 bound at least two PSTAIR-containing proteins and at least one protein was apparently not subject to tyrosine phosphorylation/dephosphorylation during the *nim*T23 block release (Figs. 4 and 5), we measured H1 kinase activity in p13 immunoprecipitates isolated from control *nim*T23 and AlcCaM/T23 cells before and after release of the *nim*T23 arrest. As expected, the p13 associated H1 kinase activity in control *nim*T23 cells increased five- to sixfold following release into mitosis (Fig. 7 *A*). But to our surprise, when calmodulin levels in the AlcCaM/T23 cells were reduced to 5% of the control *nim*T23 cells, the increase in the p13 H1 kinase activity was not substantially affected either at the block or after the release, as compared to the appropriate control (Fig. 7 *A*). Furthermore, when the *nim*T23 cells were arrested in G2 and released from the block in the presence of 2 nM extracellular Ca^{2+} as described previously, the p13 bead-associated H1 kinase activity also increase similarly to that in the *nim*T23 cells incubated in normal Ca^{2+} media (Fig. 7 *B*). When the nontemperature-sensitive R153 strain was used, there was no significant change in the p13 precipitable H1 kinase activity associated with the downshift in temperature (data not shown), as shown previously (Osmani et al., 1991*a*), indicating that the observed increase in the H1 kinase is not due to the change in the culture temperature. These data, together with the finding that at least two PSTAIR-containing proteins were precipitated by p13 beads, suggest that there are multiple H1 kinases in the p13 precipitates. Although the number and nature of the p13 associated protein kinase(s) that account for the changes in H1 kinase activity remain to be determined, these data indicate that reduction of calmodulin or Ca^{2+} does not affect the changes in the activity of the H1 kinase(s) precipitated by p13 during G2/M progression even though $p34^{cdc2}$ remains tyrosine phosphorylated.

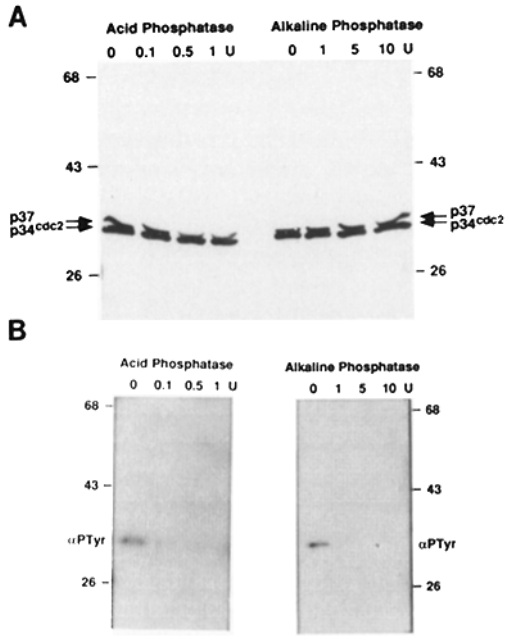

Figure 6. Phosphatase treatment of p13-binding proteins. Soluble proteins extracted from the *nim*T23 G2-arrested cells were incubated with p13 beads and then washed three times with 1 ml of HK buffer and five times with 1 ml of potato acid phosphatase buffer or calf alkaline phosphatase buffer. After incubation with the indicated amounts of potato acid phosphatase or calf alkaline phosphatase for 10 min at 30°C, two thirds of the p13-binding proteins were probed with the anti-PSTAIR antibody (*A*) and the rest was probed with the anti-phosphotyrosine antibody (*B*), after Western blotting.

Effect of Reduced Levels of Calcium or Calmodulin on Phosphorylation of the Majority of MPM-2-Reacting Proteins following Release of the nim*T23 Arrest*

The observation that low Ca^{2+} or calmodulin does not affect

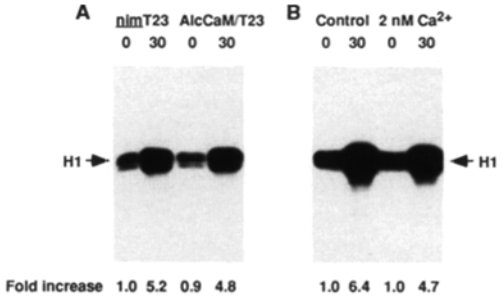

Figure 7. Effect of reduced calmodulin or Ca^{2+} levels on the histone H1 kinase activity associated with p13 beads after release from *nim*T23 arrest. (*A*) Effect of reduced calmodulin levels. The AlcCaM/T23 and *nim*T23 strains were arrested in G2 in repressing medium at 42°C and then at 25°C for 30 min. 100 μg of soluble proteins were incubated with p13 beads and the precipitated kinase activity was assayed using histone H1 as a substrate. (*B*) Effect of reduced Ca^{2+} levels. The *nim*T23 cells were arrested at 42°C in G2 in the presence of normal or 2 nM Ca^{2+} concentrations, and then at 25°C for 30 min. p13-binding proteins were isolated and used to assay H1 kinase activity. The fold increase values at the bottoms of *A* and *B* represent the change in relative kinase activity obtained by counting the excised gel slides. All values shown as the mean value from two different experiments are relative to the left *0* of *A* or *B* lane which is set at *1.0*.

overall increases in H1 kinase activity suggests that the effects on the state of tyrosine phosphorylation and NIMA activity could be selective. To evaluate this possibility further, we examined phosphorylation of M phase–specific phosphoproteins using the monoclonal antibody MPM-2, that selectively reacts with M phase–specific phosphoproteins in many systems (Davis et al., 1983; Vadre et al., 1986), including *A. nidulans* (Engle et al., 1988; Osmani et al., 1991*b*). The *nim*T23 and AlcCaM/T23 cells were arrested in G2 at the restrictive temperature in repressing medium (0) and then released into mitosis for 30 min (30), as described before. Total soluble proteins were isolated and subjected to immunoblot analysis using the MPM-2 antibody. Three separate experiments were performed and the results evaluated by both the ECL detection system and the alkaline phosphatase color reaction system. Although subtle differences could be seen between the various experimental results, the overall patterns were remarkably similar. One representative immunoblot is shown in Fig. 8. When the *nim*T23 or AlcCaM/T23 cells were arrested in G2, levels of MPM-2–reacting proteins were low (Fig. 8, left *0* and right *0*). In contrast, when the *nim*T23 mutation was released, MPM-2–reacting proteins in the *nim*T23 control cells increased both qualitatively and quantitatively (Fig. 8, left *0* and *30*). The majority of these MPM-2–reacting proteins were also present in the AlcCaM/T23 cells (Fig. 8, left *30* and right *30*) in which the calmodulin level was only ~5% of that present in control cells (Fig. 3 *A*). p58 was also detected in both cells (indicated by an arrow), which has been previously shown to be selectively phosphorylated at mitosis in *A. nidulans* detected by the MPM-2 mAb (Osmani et al., 1991*b*). Similar results were also obtained when *nim*T23 cells were grown in low extracellular Ca^{2+} (data not shown). Since there were no significant differences in MPM-2 staining pattern when the nontemperature-sensitive R153 cells were downshifted from the restrictive temperature to the permissive temperature (data not shown), the changes in the MPM-2 staining pattern observed in the AlcCaM/T23 and *nim*T23 cells are not due to the temperature shift but rather due to cellular processes that are selective for mitotic progression as have been shown in many other systems. These results indicate that reducing extracellular Ca^{2+} or calmodulin levels does not lead to a general decrease in phosphorylation of proteins that are normally modified during the G2/M transition.

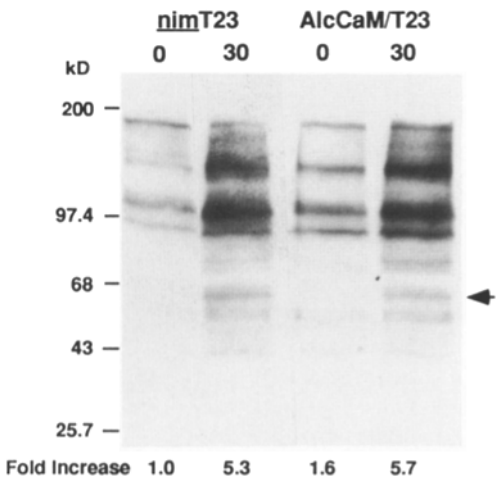

Figure 8. Effect of reduced calmodulin levels on phosphorylation of MPM-2–reacting proteins after release from the *nim*T23 arrest. The *nim*T23 and AlcCaM/T23 cells were arrested in G2 in repressing medium at 42°C (*0*) and then at 25°C for 30 min (*30*) in the presence of benomyl. Cells were harvested and ground in ER buffer and 150 μg of soluble proteins were separated by SDS gel, followed by transferring to membranes. The filters were stained with Ponceau S to insure the equal loading of proteins among lanes (data not shown), before probing with the monoclonal MPM-2 antibody. Left *0* and *30*, *nim*T23 block and release; right *0* and *30*, AlcCaM/T23 block and release. The fold increase values at the bottom represent the change in density obtained by scanning the film with an LKB Ultroscan. All values are relative to the left *0* lane which is set at *1.0*.

Discussion

In this study we have investigated the level at which Ca^{2+}/calmodulin is required for mitotic progression using conditional mutations in several G2 specific functions (the NIMA protein kinase, the NIMT tyrosine phosphatase and calmodulin) and by modulating the level of Ca^{2+} in the filamentous fungus *A. nidulans*. We demonstrate that progression into mitosis is correlated with an increase in the level of calmodulin protein although calmodulin mRNA levels do not vary. Reduction of extracellular Ca^{2+} both prevents the increase in calmodulin levels and progression of cells into mitosis. In addition, we demonstrate that by directly reducing calmodulin levels it is possible to severely impede the ability of cells to enter mitosis. To address the molecular requirements for Ca^{2+} and calmodulin for mitotic progression, the activation of two mitotic specific protein kinases have been measured when either Ca^{2+} or calmodulin levels are held at a low level. We demonstrate that a threshold level of Ca^{2+}/calmodulin is required for the activation of NIMA as a protein kinase and for the *nim*T^{cdc25}-mediated tyrosine dephosphorylation of $p34^{cdc2}$. In contrast, the activation of the mitotic specific phosphorylations detected by the MPM-2 mAb and the mitotic activation of a p13 associated histone H1 kinase are shown not to require this threshold level of either Ca^{2+} or calmodulin. These data demonstrate that Ca^{2+} and calmodulin are required for specific components of mitotic regulation in *A. nidulans* that involve the activation of NIMA by an unknown mechanism and $p34^{cdc2}$ by promoting its tyrosine dephosphorylation.

The effects of reduced levels of Ca^{2+} or calmodulin on cell cycle functions are apparently selective, if not specific, for activation of NIMA and $p34^{cdc2}$ since neither affects several other cell cycle–specific events. For instance, reduced Ca^{2+} or calmodulin levels do not affect the tyrosine phosphorylation of $p34^{cdc2}$ at the G2 arrest point of *nim*T23 even though it has been demonstrated that tyrosine phosphorylation of $p34^{cdc2}$ requires that it binds to newly synthesized cyclin B protein (Gould and Nurse, 1989; Meijer et al., 1991) and requires the activity of two protein kinases, WEE1 and MIK1 (Featherstone and Russell, 1991; Lundgren et al., 1991) which may be activated by serine/threonine dephosphorylation (Smythe and Newport, 1992). Therefore, reducing Ca^{2+} or calmodulin levels does not prevent protein synthesis, or protein kinase activity or phosphatase activity in general but it does selectively prevent activation of the

NIMA protein kinase at the *nim*T23 G2 arrest point. Furthermore, when the *nim*T23 mutation is released in the absence of normal levels of Ca^{2+} or calmodulin, the tyrosine dephosphorylation of $p34^{cdc2}$ is strongly inhibited and cells remain in G2 even though the H1 kinase activity measured using $p34^{cdc2}$ affinity p13 beads increases significantly. This result may indicate the existence of non-$p34^{cdc2}$ protein kinase activity on the p13 beads. We have observed that two anti-PSTAIR-reacting proteins in our p13 precipitates which are not apparently two different phosphorylated forms of $p34^{cdc2}$. The larger of these proteins does not contain phosphotyrosine based on the lack of interaction with anti-phosphotyrosine antibodies. If the increase in H1 kinase activity after release of the *nim*T23 arrest in the absence of normal levels of Ca^{2+} or calmodulin is due to this second PSTAIR-containing protein, then it must be activated either indirectly by NIMT or by the ability of NIMT to carry out nonphosphotyrosine dephosphorylation since CDC25 protein has been shown to harbor protein serine phosphatase activity in vitro (Millar et al., 1991). The possibility also exists that other non-$p34^{cdc2}$–related protein kinases could be present in the p13 precipitates as it has been recently shown that MAP kinases can bind to this affinity matrix (Shibuya et al., 1992). Finally, the correlation between tyrosine dephosphorylation of $p34^{cdc2}$ type protein kinases and activation as an H1 kinase is not always observed. In budding yeast, it has been shown that increased H1 kinase associated with $p34^{cdc28}$ can occur without apparent tyrosine dephosphorylation of $p34^{cdc28}$ (Sorger and Murray, 1992; Amon et al., 1992). Therefore, there are numerous possible explanations that could account for our observation that H1 kinase activity increases in the absence of tyrosine dephosphorylation when the concentration of Ca^{2+} or calmodulin is held low. The pertinent observation is that some protein phosphorylation detected by the MPM-2 antibody can occur when *nim*T23 is released in the absence of normal levels of Ca^{2+} or calmodulin but the activated (partially?) NIMT cannot dephosphorylate $p34^{cdc2}$ phosphotyrosine in the normal manner. The level at which Ca^{2+} and calmodulin are required for tyrosine dephosphorylation could therefore be via a modification of either NIMT, $p34^{cdc2}$ or the ability of these components to interact correctly. It is not, however, due to some general inability of the cell to function correctly.

In addition, the level of the phosphoproteins detected by the MPM-2 mAb was not significantly modified by the absence of normal levels of Ca^{2+} or calmodulin when the *nim*T23 mutation was released. This indicates that some mitotic specific protein phosphorylation is not affected by the reduction in the level of Ca^{2+} or calmodulin and that NIMT can become activated sufficiently under conditions of low Ca^{2+} or calmodulin to allow the MPM-2 detected phosphorylation to occur. It is therefore unlikely that $p34^{cdc2}$ is directly responsible for the MPM-2–detected phosphorylation but perhaps the second kinase(s) present on the p13 beads that is activated downstream of $nimT^{cdc25}$ in the absence of Ca^{2+} or calmodulin is the "MPM-2" kinase.

Very little is known concerning the mechanism by which Ca^{2+} and calmodulin are involved in activation of NIMA and NIMT. Ca^{2+}/calmodulin could directly interact with and activate NIMT and/or NIMA. Alternatively, NIMT and/or NIMA activities could be modulated as a consequence of activation of other Ca^{2+}/calmodulin-dependent enzymes in a protein phosphorylation/dephosphorylation cascade. Since calmodulin neither binds directly nor activates purified NIMT or NIMA in the presence of Ca^{2+} (Lu, K. P., S. A. Osmani, and A. R. Means, unpublished data), the former possibility is unlikely. We favor an indirect pathway since recent evidence indicates that the cdc25 protein may require serine/threonine phosphorylation for its activity (Kumagai and Dunphy, 1992; Izumi et al., 1992). In addition, we have found that an active form of NIMA is a phosphoprotein both in *A. nidulans* or when expressed in bacteria and that serine/threonine phosphorylation is essential for its enzymatic function (Lu et al., 1993). Since the dephosphorylated form of NIMA cannot be activated by autophosphorylation in vitro (Lu et al., 1993), we suspect NIMA to be an intermediate component in a cascade of protein kinases, as is the case for MAP kinase (for review see Thomas, 1992). Therefore, we suggest that Ca^{2+}/calmodulin is involved in regulating NIMA and NIMT via activation of Ca^{2+}/calmodulin-dependent enzyme(s).

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