BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 204–208 (1997)
ARTICLE NO. RC976253

# Role of the Fission Yeast nim1 Protein Kinase in the Cell Cycle Response to Nutritional Signals

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Received January 27, 1997

**The fission yeast cdr1/nim1 protein kinase phosphorylates and inactivates the wee1 cdc2-inhibitory kinase. We have investigated the role played by cdr1/nim1 in the connection between nutritional signals and the cell cycle machinery. We show that loss of nim1 activity impairs the appropriate cellular adaptation to nutritional changes. However, the reduction in cell size at division in response to nitrogen starvation is independent of nim1. Moreover, we report that nim1 is an unstable protein that is rapidly degraded upon starvation, through a mechanism that is dependent upon protein synthesis. We propose that nim1, as a constitutive indirect activator of cdc2 at mitosis, favors the cellular response to starvation but does not actively participate in it. On the contrary, upon nitrogen starvation nim1 must be actively destroyed to protect the cells from a commitment into the cell cycle under unfavourable growth conditions.** 

In fission yeast, control of the cell cycle is connected to the nutritional status through a signalling pathway that appears to feed ultimately into the cdc2 activity regulatory network (1-3). However, little is know about the identity of the proteins that transduce nutritional cues to the cell cycle regulators. One of the major candidates for such a regulator is the nim1/cdr1 serine-threonine protein kinase. The identification of the *Schizosaccharomyces pombe* nim1+/cdr1+ gene was achieved through two independent genetic approaches. First, the nim1+ gene (new inducer of mitosis) was identified as an extragenic suppressor of a cdc25 thermosensitive mutation (4). As does the cdc25 phosphatase, nim1 acts as a dose dependent inducer of mitosis. Upon nim1 overexpression, cells enter mitosis at a reduced size compared to the wild type. Nim1 gene disruption is viable but the cells divide with an elongated phenotype (4, 5), indicating that nim1 function probably operates permanently during the cell cycle. Second, mutants called cdr1 and cdr2 (changed division response) that are affected in their response to nutrient limitation were identified (6). The cdr1 gene was cloned by plasmid rescue of a conditional lethal cdr1-76 cdc25-22 double mutant and found to be allelic to nim1 (5). *In vitro,* nim1/cdr1 phosphorylates and inactivates the wee1 kinase thus leading to the activation of a cdc2/cyclin complex (7, 8). This is in agreement with genetic studies that have shown that nim1/cdr1 is epistatic to wee1 and may act as a negative regulator of it (4). Wee1 is hyperphosphorylated in fission yeast cells that overproduce nim1 (9). Likewise, wee1 phosphorylation is reduced in cells in which the nim1 gene has been disrupted (9). These observations, together with the fact that nim1 seemed to be involved in the control of the cell cycle in response to nutritional signals, prompted us to examine its role and its regulation.

## MATERIAL AND METHODS

*Yeast strains, cultures, and cytological observations.* The *S. pombe* strain Q648 (h+ cdr1::ura4+ ura4.D18 leu1.32)(4) was transformed with the pIRT2 or the pREP1 derived plasmids indicated below. The nmt1 inducible promoter was kept under repressed conditions by addition of 4 $\mu$M thiamine (10, 11). Derepressed conditions were obtained by washing the cells three times in minimal medium and growing them in the absence of thiamine during 15 hours. For the nitrogen starvation experiments, cells cultivated in minimal medium were washed three times and transferred to minimal medium without ammonium chloride. Thermoresistance was assayed as described (12). Cytological observations and flow cytometry analyses were performed as described (13).

*Plasmids.* A NdeI site was inserted at the initiating methionine and the nim1 cDNA was cloned downstream the nmt1 regulatable promoter between the NdeI and BamH1 site of the pREP1 vector. Inactive nim1 kinase was obtained by substitution of the lysine 41 to alanine (nim1K41A). To construct HA tagged version of nim1 and nim1Δ354-589, the nim1 cDNA was digested with BglII (nt 1638) or HindIII (nt 1063) respectively and after mung bean nuclease or Klenow polymerase treatment a double HA tagged was inserted.

*Protein extracts and immunoblotting.* Yeast cells were harvested by centrifugation and quickly broken with cold 500 $\mu$m glass beads

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0006-291X/97 $25.00

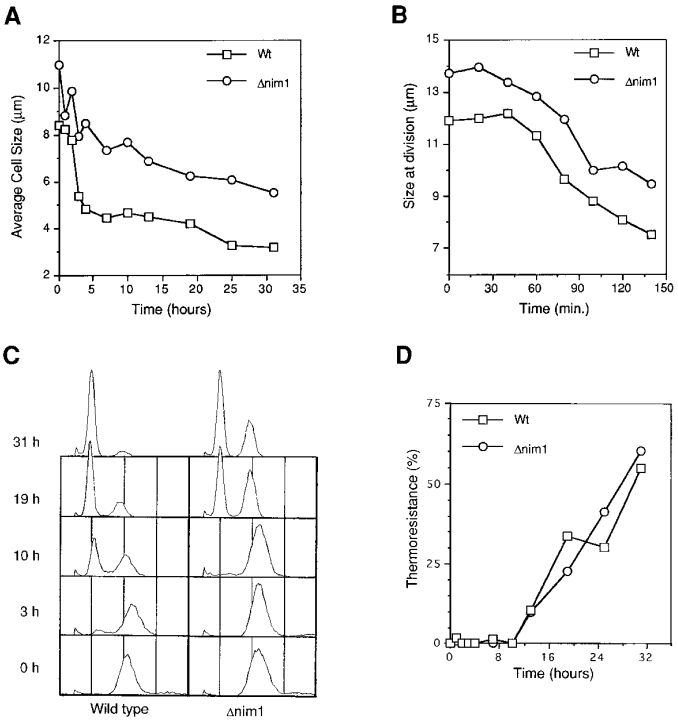


**FIG. 1.** Effects of nitrogen starvation on the cell cycle parameters in wild-type and nim1/cdr1 mutant *Schizosaccharomyces pombe* cells. Exponentially growing populations of either wild-type (Wt) or nim1/cdr1 disruptant *S. pombe* cells (Δnim1) in minimal medium were washed and re-inoculated in nitrogen free medium as described in the methods section. Samples were taken at intervals to determine microscopically the average cell size (A) or cell size at mitosis by measurement of a minimum of fifty cells (B), the DNA content by flow cytometry (C) and the thermoresistance (D). In (C) the two peaks represent G1 and G2 cells containing a 1C and 2C DNA content respectively.

in lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 mM DTT and 0.1% triton X100) containing the following protease inhibitors: 0.1 mM PMSF, 5 $\mu$g/ml leupeptin, 20 $\mu$g/ml soybean trypsin inhibitor, 2 $\mu$g/ml aprotinin, 80 $\mu$g/ml TLCK, 40 $\mu$g/ml TPCK, 1$\mu$g/ml pepstatine and 5 and 10 $\mu$g/ml calpain inhibitor I and II respectively, and the following phosphatase inhibitors: 50 mM sodium fluoride, 0.1 mM sodium orthovanadate and 10 mM pNPP. The soluble protein fraction was recovered by three centrifugations of 5 min at 17,000g. The samples were electrophoresed on SDS-polyacrylamide gel and immunoblots were probed using monoclonal antibodies raised against the HA epitope or rabbit polyclonal antibody raised against *S. pombe* cdc2.

## RESULTS

### *Involvement of nim1/cdr1 in the Response to Nitrogen Starvation*

The effect of nitrogen starvation on cell size and cell cycle distribution were first investigated on wild-type cells. Upon transfer to nitrogen free medium average cell size rapidly decreased (Figure 1A). This effect was particularly obvious after three hours of starvation with about 40% reduction. Accordingly, average cell length at division rapidly decreased from 11.9 $\mu$m to 7.8 $\mu$m within the two first hours following starvation (Figure 1B). Given the relative duration of the different phases of the fission yeast cell cycle, exponentially growing wild type fission yeast cells are predominantly in the G2-phase and the G1- and S-phases are therefore usually not detected by flow cytometry. As shown in figure 1C, upon nitrogen starvation the percentage of wild type cells in G1 progressively increased with the G2-phase becoming correspondingly shortened. Indeed, cells that enter mitosis at reduced size must increase the duration of G1 to reach the critical size that is required to enter S-phase (14). Up to 10 hours after transfer to nitrogen free media, the cells were still proliferating, as monitored by cell counting (not shown) and by the presence of an S-phase (Figure 1C). At 31 hours the S-phase was undetectable (Figure 1C), indicating that the cells stopped proliferating. Exit from the cell cycle was demonstrated by an increase in thermoresistance (15) that was observed only after 10 hours of nitrogen withdraw and which was up to 50% after 31 hours (Figure 1D). This exit in a G0 quiescent state was achieved with a G1 DNA content (Figure 1C).

When grown in minimal medium, the average length of a nim1/cdr1 disruptant cell was 30% longer than a wild type cell (Figure 1A). In response to nitrogen starvation a reduction in average cell length was again observed (Figure 1A). However, the nim1 disruptants take more than four time longer than wild type cells to exhibit a similar 40% reduction in length and this shortening was progressive and irregular. Nonetheless, the average cell length at division shared a similar kinetic of reduction in both wild type and mutant cells (Figure 1B). Flow cytometry analysis showed that the increase in G1-phase of nim1/cdr1 disruptant cells was not detected until 10 hours after nitrogen starvation as compared to 3 hours in wild type cells (figure 1C). This results reflects the fact that, although the size at division is reduced in mutant cells, they remain longer at a size that is larger than that required to enter S-phase. Nim1/cdr1 disruptant cells ceased proliferating with either a G1 or a G2 DNA content (Figure 1C) and were likely to be stopped in a G0 stage since their thermoresistance was found to be comparable to the wild type strain (Figure 1D). From these data we can conclude that nim1 is not required for the reduction of the cell size at division in response to nutrient starvation. However, in a strain lacking a functional nim1 protein, the efficiency of the physiological response, i.e. the arrest in G1-phase, is delayed and impaired.

Whether the catalytic activity of the nim1 protein kinase was involved in the efficiency of the response to nitrogen starvation was investigated. A nim1/cdr1 disruptant strain was transformed with a plasmid allowing the expression of this kinase under the control of a thiamine repressible promoter. Both an active and a catalytically inactive kinase (mutation of lysine 41 to

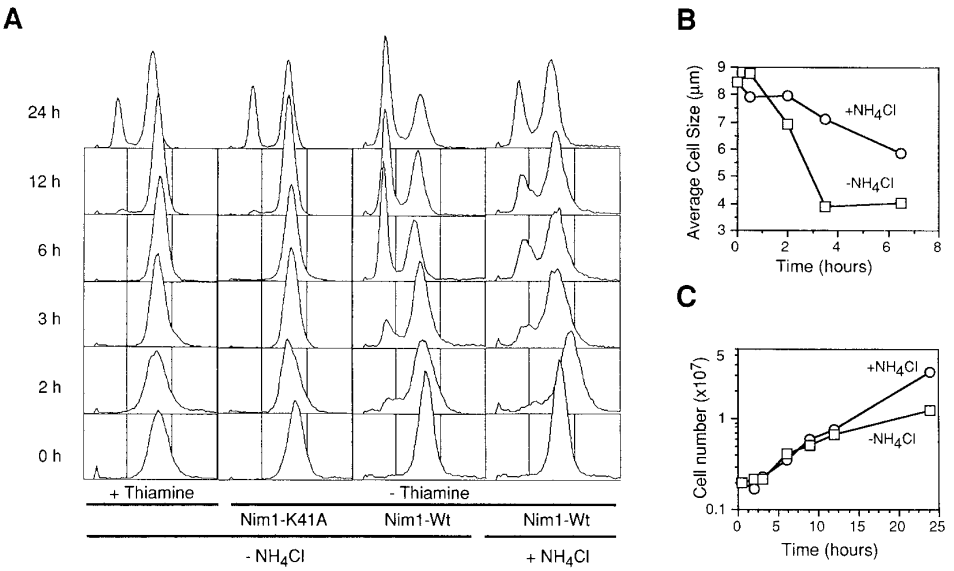


**FIG. 2.** Nutritional response requires nim1 catalytic activity. Nim1/cdr1 disruptant strain was transformed with plasmids allowing the regulated expression of nim1 either in an active (Wt) or in an inactive form (K41A mutation). Cells grown in the presence of thiamine, or shifted for 15 hours in thiamine-free media (to allow nmt1 promoter expression), were transferred, in the presence or in the absence of thiamine, to nitrogen-free medium ($-NH_4Cl$) or to nitrogen-containing medium ($+NH_4Cl$). Cell cycle distribution was determined by flow cytometry. Average cell size (B) and cell number (C) were determined microscopicaly in the presence or in the absence of ammonium chloride ($NH_4Cl$) in nim1/cdr1 disruptant cells expressing wild type nim1.

alanine) were used. After induction of the expression of these proteins by removal of thiamine from the culture media for 15 hours, the cells were transferred in nitrogen free media and their behaviour examined. As shown in figure 2A, the cell cycle distribution of cells expressing a catalytically inactive nim1 kinase was similar to that of cells that did not express nim1. In contrast, expression of an active nim1 kinase rescued the ability of the nim1/cdr1 disruptant strain to respond efficiently to nitrogen starvation, i.e. rapid changes in cell cycle distribution (figure 2A) and abrupt shortening of the average cell size (Figure 2B). These results indicate that the nim1/cdr1 catalytic activity is necessary for an efficient cellular response to nitrogen starvation.

Because of the known function of nim1 as a mitotic inducer, we also performed an experiment of nim1 overexpression in cells grown in nitrogen containing media. In this case, a modification in cell cycle distribution, with detection of cells in G1, was also observed. However, flow cytometry showed a peak of G1 cells that was not sharp but rather merged with the G2 peak indicating the presence of a large population of cells in S-phase (figure 2A). Accordingly, the average cell size also decreased upon nim1 overexpression, but to a lower extent and with kinetics that were different to that in the nitrogen-free medium (figure 2B). Hence, in the presence of a nitrogen source, although the size of daughter cells is reduced, these cells remain able to reach the minimal size that is required to enter S-phase and therefore to proliferate (Figure 2C). Accordingly, in nitrogen containing media, the thermoresistance of the cells overexpressing nim1 remained not higher than 7%. These results suggest that while the efficiency of the cellular response to the lack of nitrogen results from the mitotic inducer effect of nim1, an additional event operating at G1/S prevents an unappropriate commitment to the mitotic cycle upon lack of nutrients.

### *Regulation of nim1 upon Nitrogen Starvation*

All our attempts to detect convincingly the nim1/cdr1 protein kinase by western-blot in wild type *S. pombe* cell extracts were unsuccessful. We therefore made use of cell overexpressing various nim1 constructs under the control of the nmt1 regulatable promoter to investigate the behaviour of this kinase upon nitrogen starvation. All forms of the kinase that were used in that study were epitope-tagged at the carboxy-terminal end with two HA motifs in tandem and monoclonal antibodies were used to detect them. The ability of these modified proteins to fully replace the wild type sequence for a normal response to nitrogen starvation and for the complementation of a cdc25 thermosensitive allele was checked and found to be identical to the untagged form (data not shown).

Upon transfer of cell overexpressing the cdr1/nim1 kinase into nitrogen free media, the nim1 protein level rapidly decreased and was barely detectable within 3 to 5 hours (Figure 3A). In these conditions the cdc2

protein level remained constant (Figure 3A). In nitrogen containing media, as a consequence of the promoter induction, a continuous accumulation of nim1 was detected (Figure 3A). In an attempt to estimate the nim1 protein stability, we monitored its level after protein synthesis inhibition (Figure 3B). In the presence of nitrogen and upon treatment with cycloheximide, the nim1 protein did not accumulated. In nitrogen free media, the decrease of nim1 protein level was inhibited in the presence of cycloheximide and its level remained constant during the 5 hours of the experiment. These results demontrate that nim1 is degraded upon nitrogen starvation through a mechanism that is dependent on protein synthesis. In addition, the nim1 regulatory domain appears to be essential for this degradation process since a version of nim1 that lacks residues 354 to 594 was not degraded after five hours of nitrogen starvation (Figure 3C).

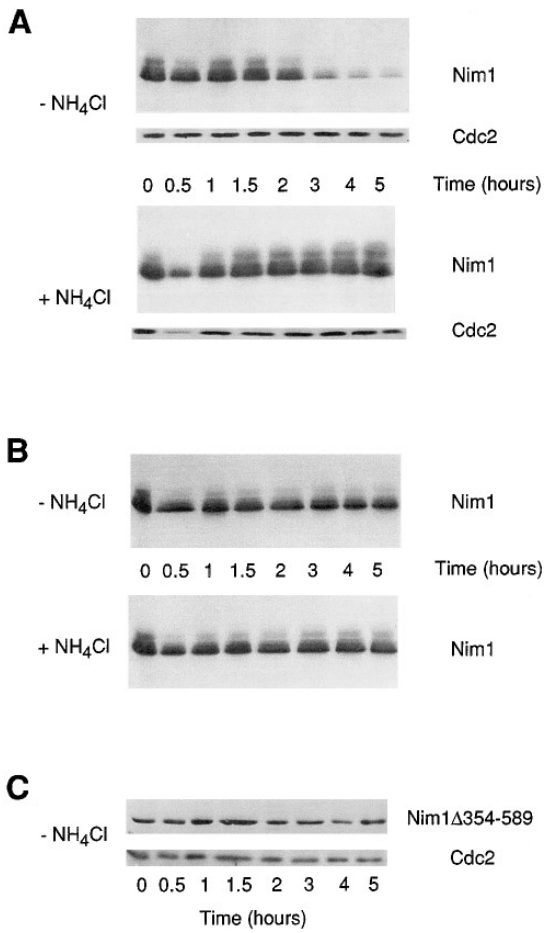


**FIG. 3.** Nim1 degradation upon nitrogen starvation (A, B) *S. pombe* cdr1/nim1 disruptant cells expressing HA epitope tagged nim1 under the control of the nmt1 thiamine repressible promoter (15 hours of promoter induction) were washed and transferred to medium lacking or containing ammonium chloride ($NH_4Cl$) in the absence (A) or in the presence (B) of 200 $\mu$g/ml cycloheximide (CHX). (C) A HA-tagged truncated form of nim1 that is restricted to the catalytic domain (residues 1 to 354) was expressed in nim1 disruptant cells before transfer to nitrogen free media as described in (A). The upper bands recognised by the anti-HA monoclonal antibody in panel (A) and (B) are phosphorylated forms of nim1 (unpublished data).

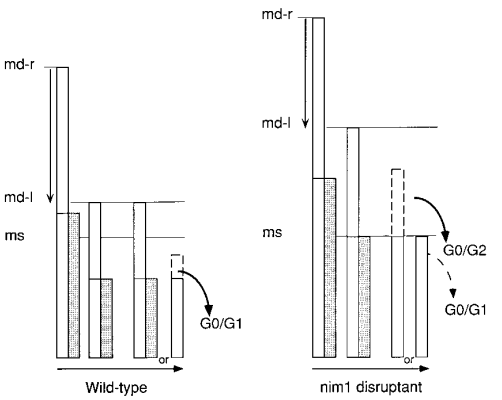


**FIG. 4.** A model for the behaviour of wild type and nim1 disruptant cells upon shift to nitrogen-free media. A *S. pombe* cell (white bars) divides into two identical daughter cells (gray bars) that are roughly half of the size of the mother cell. The cells must have reach a minimum size (ms) to enter S-phase (14) (18). Usually, newborn wild type cells growing in rich medium are already larger than the "ms" requirement and immediately enter S-phase after cytokinesis. At G2/M the cell must reach a minimal size to divide (md) (19). This requirement is higher in rich medium (md-r=12$\mu$m) than in low nitrogen medium (md-l=6.5$\mu$m). Upon transfer of wild type cells to nitrogen free medium (horizontal arrow), daughter cells from the first division are larger than "ms" and only have to grow a little to reach "md-l" and to themselves divide. This division gives two daughter cells that are smaller than "ms". These may either reach "ms" and then be commited to the cell cycle, if nutrients are sufficient, or be unable to reach "ms" and therefore exit the cell cycle with a G1 DNA content. In cdr1/nim1 disruptant cells, since "md-l" is large (9.5 $\mu$m versus 14 $\mu$m for md-r), starved daugther cells will stay for a longer time larger than "ms" and therefore will immediatly enter S-phase. Finally, because of the lack of nutrients required to reach "md-l", cells will become unable to divide and a number of them will exit the cell cycle with a G2 DNA content. Those cells that succeed in dividing will arrest their cycle with a G1 DNA content.

## DISCUSSION

Our kinetic analyses of the average cell size reduction and subsequent changes in cell cycle parameters demonstrated that nim1 disruptant cells do not efficiently exit from the cell cycle in G1 in response to nitrogen starvation. From these observations and others (2, 6), it could be thought that nim1 would directly modulate cell cycle control in response to nutritional signals. However, we also report here that nim1 is not necessary for the size decrease at division immediately observed upon transfer into nitrogen-free medium. These conflicting results lead us to propose a model in which the nim1 kinase, through its activatory function at mitosis, ensures that the cell cycle parameters are correctly set up for an efficient cellular response to the lack of nitrogen (figure 4). We have shown here that

nim1 is rapidly degraded upon nitrogen starvation. Within three hours of the change in growth medium, and despite continous synthesis, driven by the nmt1 promoter, nim1 becomes barely detectable by Western blotting. In similar experiments we shown that cdc2 protein level remain constant (see also (16)). Nim1 degradation is tightly dependent upon protein synthesis and is blocked when the cells are treated with cycloheximide. This unexpected finding indicates that the active degradation mechanism that is triggered by nutritional signals requires *de novo* protein synthesis. The nature and the identity of the regulator(s) whose synthesis is required for nim1 degradation remain to be investigated. While nim1 was rapidly degraded upon nitrogen starvation, we found that the truncated version of nim1 was more stable, suggesting that residues located in the C-terminal domain are important to this unstability. A computer search for PEST instability sequences (17) revealed that candidate domains with the more significant scores are all located in the carboxy-terminal half of nim1, within residues 417 to 528. It has been proposed that PEST-rich regions confer susceptibility to rapid intracellular proteolysis of a given protein and that removal of them should reduce its turnover (17). Our results reported here suggest that nim1 instability and rapid degradation upon nutritional starvation might be dependent upon the presence of PEST sequences that lie in the C-terminal end of the protein.

Why should nim1 be degraded within a short time after nutritional starvation? Although a G1 wee1-like function has not been yet identified in fission yeast, this observation is consistent with an activatory function of nim1 on the G1 form of the cdc2 kinase. The rapid degradation of nim1 upon nitrogen starvation would be therefore essential to prevent entry into S-phase, avoiding a commitment into the cell cycle under unfavourable growth conditions.

## ACKNOWLEDGMENTS

We gratefully acknowledge F. Goubin for nim1 mutagenesis, W. Dunphy, P. Léopold, P. Russell and P. Young for the generous gift of strains, plasmids and reagents, J. Hyams for valuable advises and comments on the manuscript, and the members of our lab for helpful discussions and suggestions. We are particularly grateful to V. Baldin for her interest and participation to this project. This work was supported by le CNRS, l'université Paul Sabatier, l'Association pour la Recherche sur le Cancer, la Ligue Nationale Contre le Cancer and la Ligue Contre le Cancer comité de la Dordogne.

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