Proteolysis and Tyrosine Phosphorylation of p34<sup>cdc2</sup>/Cyclin B

THE ROLE OF MCM2 AND INITIATION OF DNA REPLICATION TO ALLOW TYROSINE PHOSPHORYLATION OF p34<sup>cdc2</sup>

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Previously, it has been shown that <i>Aspergillus</i> cells lacking the function of nimQ and the anaphase-promoting complex (APC) component bimE<sup>APC1</sup> enter mitosis without replicating DNA. Here nimQ is shown to encode an MCM2 homologue. Although mutation of nimQ<sup>MCM2</sup> inhibits initiation of DNA replication, a few cells do enter mitosis. Cells arrested at G<sub>1</sub>/S by lack of nimQ<sup>MCM2</sup> contain p34<sup>cdc2</sup>/cyclin B, but p34<sup>cdc2</sup> remains tyrosine dephosphorylated, even after DNA damage. However, arrest of DNA replication using hydroxyurea followed by inactivation of nimQ<sup>MCM2</sup> and bimE<sup>APC1</sup> does not abrogate the S phase arrest checkpoint over mitosis. nimQ<sup>MCM2</sup>, likely via initiation of DNA replication, is therefore required to trigger tyrosine phosphorylation of p34<sup>cdc2</sup> during the G<sub>1</sub> to S transition, which may occur by inactivation of nimT<sup>cdc2</sup>. Cells lacking both nimQ<sup>MCM2</sup> and bimE<sup>APC1</sup> are deficient in the S phase arrest checkpoint over mitosis because they lack both tyrosine phosphorylation of p34<sup>cdc2</sup> and the function of bimE<sup>APC1</sup>. Initiation of DNA replication, which requires nimQ<sup>MCM2</sup>, is apparently critical to switch mitotic regulation from the APC to include tyrosine phosphorylation of p34<sup>cdc2</sup> at G<sub>1</sub>/S. We also show that cells arrested at G<sub>1</sub>/S due to lack of nimQ<sup>MCM2</sup> continue to replicate spindle pole bodies in the absence of DNA replication and can undergo anaphase in the absence of APC function.

Promotion of mitosis is controlled by coordinate activation of the p34<sup>cdc2</sup>/cyclin B (1, 2) and NIMA protein kinases (3, 4). In many systems, the key step for mitotic initiation in G<sub>2</sub> is Tyr-15 phosphorylation of p34<sup>cdc2</sup>/cyclin B (5–11) to cause activation of this kinase complex (known as mitosis-promoting factor or MPF<sup>‡</sup>). Rapid tyrosine dephosphorylation of p34<sup>cdc2</sup> occurs by tipping the balance between the Wee1 kinase and the Cdc25 phosphatase to favor dephosphorylation during G<sub>2</sub>/M (9, 11).

During S phase and G<sub>2</sub>, tyrosine phosphorylation of p34<sup>cdc2</sup> acts to prevent premature entry into mitosis, particularly after DNA damage or when DNA replication is perturbed (12–17), although this level of regulation is lacking in Saccharomyces cerevisiae (18, 19).

Exit from mitosis is controlled by proteolysis (20–23) of key mitotic regulatory proteins mediated by the anaphase-promoting complex (APC) or cyclosome (24–29). The APC is thought to promote degradation of proteins such as Pds1 (30–32) and Cut2 (33) to assist anaphase, and then cyclin B to allow exit from mitosis into G<sub>1</sub> (21–23, 34). Components of the APC have also been implicated in interphase cell cycle control (13, 35, 36).

During progression into G<sub>1</sub> and S phase, cell cycle-specific proteolysis and tyrosine phosphorylation of p34<sup>cdc2</sup> need to be coordinated in some way to ensure that mitosis does not occur during G<sub>1</sub> or before DNA replication has been completed. For instance, if the APC failed to function during late mitosis or G<sub>1</sub>, then accumulation of cyclin B could potentially form a complex with p34<sup>cdc2</sup> and, if the balance between Wee1 and Cdc25 still favors dephosphorylation of p34<sup>cdc2</sup>, MPF could be generated and so promote cells back into a mitotic state. Therefore, during the transition from metaphase to interphase, a coordinated inactivation of APC activity (to allow accumulation of cyclin B) and re-activation of Tyr-15 phosphorylation of p34<sup>cdc2</sup> (to prevent premature accumulation of MPF activity) must occur.

In the current study, we have asked how tyrosine phosphorylation of p34<sup>cdc2</sup> and proteolysis mediated by the APC are coordinated to allow normal progression from G<sub>2</sub> into M and then from M into G<sub>1</sub> and S phase. Our studies indicate that during G<sub>1</sub> the function of the APC helps prevent progression into mitosis. The function of nimQ<sup>MCM2</sup>, a homologue of MCM2 (37) that plays a key role in licensing DNA for a single round of replication per cell cycle (38–41), is shown to allow transfer of checkpoint control over mitotic initiation from the APC to include tyrosine phosphorylation of p34<sup>cdc2</sup> upon initiation of DNA replication. Our data further show that cells lacking the nimQ<sup>MCM2</sup> function undergo multiple rounds of spindle pole body (SPB) duplication in the absence of the chromosomal cycle. The molecular mechanisms proposed help explain the logic of the cell cycle in organisms regulating mitotic initiation by tyrosine dephosphorylation of p34<sup>cdc2</sup> and mitotic exit via the APC.

EXPERIMENTAL PROCEDURES

Reciprocal Shifts and [<sup>3</sup>H]Adenine Labeling Studies—To determine the precise cell cycle arrest point of the ts lethal nimQ20 mutation, we performed reciprocal shift experiments (42); however, instead of monitoring nuclear division, we measured DNA replication using [<sup>3</sup>H]adenine to label cells of strain SWJ186 (nimQ20, choA1) adapted from Ref. 43. The experiment was reproduced two times with the same results.

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Aspergillus Transformations and Libraries and cDNA Isolation—For cloning and isolation of nimQ, transformation of Aspergillus was performed as described (44). Analysis of transformants, media for propagation and genetic analysis of Aspergillus nicQ mutants were as described previously (35, 45, 46). cDNA clones were isolated from a library constructed from agar GT1 (46). Standard procedures for recombinant DNA manipulations were used (47). DNA sequencing was performed on single-stranded templates using a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp.). nimQ was cloned by complementation of the nimQ20 ts-lethal mutation, taking advantage of its previously mapped position on linkage group VII (48). The wild-type nimQ gene was isolated from a cosmid library of 468 independent LGVII-specific clones (49) obtained from the Fungal Genetics Stock Center. Cosmid DNA from five pools of approximately 95 individual clones each (provided by J. H. Doonan) were cotransformed into a nimQ20, argB2 strain (SWJ028; nimQ20, argB2, pabaA1, wA2) using the A. nidulans autonomously replicating helper plasmid pDHD25 (50) to eventually identify a single clone, W18C11, capable of fully complementing nimQ20. The complementing DNA was localized (51) to a 3.6-kb HindIII-KpnI subclone. Using this subclone as a probe, eight cDNA clones were isolated and purified. Six of the cDNAs appeared to be related, as they shared at least two out of three EcoRI restriction fragments, and the two longest cDNAs (each ∼3.1 kb) were able to fully complement the ts-lethality of nimQ20 as phage clones or after subcloning into pBluescript KS+ and KS− (Stratagene). Partial restriction analysis of the cDNA clone into its own locus complement the mutant allele, whereas heterologous integration of a promoterless cDNA does not. The ability of the cDNAs to fully complement nimQ20 at high frequency (n = 228 total nimQ1 transformants from four experiments, using approximately 5 μg of DNA in each transformation) confirms that we had cloned the nimQ gene. The two remaining cDNAs appeared unrelated to the others. Restriction endonuclease mapping with nine different enzymes showed that the 3.1-kb complementing cDNA clones closely matched the 3.6-kb HindIII-KpnI genomic clone in a 1.7-kb region where they overlapped. Based upon comparison of the genomic restriction map with the cDNA sequence, the genomic clone harbors part of the gene, and is predicted to lack the C-terminal 334 amino acid residues. A stable transformant that fully complemented nimQ20 and argB2 was made by co-transferring this partial genomic clone and pDH25 helper plasmid into a nimQ20, argB2 strain (SWJ028). Transformants were then outcrossed to a wild-type strain, and analyzed for recovery of nimQ20 ts-lethal phenotype. Various other genetic markers for spore color and nutritional auxotrophies showed the expected 1:1 segregation in these crosses. However, no ts-lethal segregants were recovered from among 157 progeny, indicating tight linkage between the nimQ chromosomal locus and the integrating genomic DNA, again confirming that we had cloned nimQ.

Aspergillus Strains and General Techniques—A. nidulans strains used in this study included R153 (pyroA4, wA3), SO4 (bimE7, pabaA1, wA2), SO53 (nimT23, wA2), SO54 (nimA5, wA2), SWJ028 (nimQ20, argB2, pabaA1, wA2), SWJ145 (nimQ20, bimE7, methB3, choA1), SWJ176 (nimQ20, choA1), SWJ280 (nimP22, pyroA4, choA1), and SWJ292 (nimP19, nicA2). Media and techniques for growing Aspergillus cells, temperature shifts, protein extraction, immunoprecipitation, Western blotting for NIMQ (B. Y. Nim et al., manuscript in preparation), and Ty-fornophosphorylation of p34CDC2, p34cdc25 H1 kinase assay, and 4′-6-diamidino-2-phenylindole (DAPI) staining for chromosome mitotic index determination were all as described previously (13, 45, 52–56).

Indirect Immunofluorescence Staining of Microtubules and SPBs—The procedures for indirect immunofluorescence staining of microtubules in A. nidulans using a mouse monoclonal anti-tubulin antibody (B5-1-2, Sigma) were as described previously (13, 54). The secondary antibody was goat anti-mouse IgG conjugated with fluorescein isothiocyanate (P9006, Sigma) used at 1:100 dilution. To stain SPBs, affinity-purified anti-γ-tubulin antisera were used as described previously (57). Secondary antibody for γ-tubulin staining was sheep anti-rabbit IgG Cy3 conjugate (C2036, Sigma) used at 1:200 dilution. Examination and photography were made using a Nikon (Eclipse E800) microscope.

RESULTS

nimQ Is Required for DNA Replication and Encodes an MCM2 Homologue, a Component of the DNA Replication Licensing System—Previous flow cytometric studies of nimQ20-arrested conidia (unincubated dormant spores) germinated in minimal media revealed a general defect in DNA synthesis (35). To characterize the arrest of nimQ20 cells grown in complex media, we labeled cells in vivo with [2,8-3H]adenine (43) and measured the synthesis of labeled DNA at 43 °C. Conidial spores containing nimQ20 germinated at permissive temperature underwent three rounds of DNA synthesis and began a fourth during the 8-h experiment (Fig. 1A). By contrast, essentially no DNA synthesis occurred at the restrictive temperature (Fig. 1A). The very slight and gradual increase in DNA labeling observed is likely to be mitochondrial DNA synthesis that can occur without nuclear DNA replication during germination (53).

We also analyzed whether nimQ function was required for continued DNA synthesis after arrest of DNA replication using hydroxyurea (HU) in a reciprocal shift analysis (42). Conidia containing nimQ20 grown at 30 °C (permissive temperature) in the presence of 20 mM HU were unable to synthesize DNA and were also incapable of DNA synthesis after removal of the HU and transfer to 43 °C (restrictive temperature) (data not shown). The analysis indicates that nimQ is required for the initiation of DNA replication and for continued DNA replication after the HU arrest point.

We cloned nimQ by complementation and isolated a cDNA that was then sequenced (data not shown, GenBank™ accession no. AF014813). The cDNA encodes a putative open reading frame of 889 amino acids corresponding to a protein of 99.95 kDa, assuming that the first in-frame methionine is used for translational initiation. An in-frame stop codon occurs upstream of the putative initiation codon (at base pair 25), indicating that the coding region does not extend beyond the 5′ end of the clone. A data base search revealed strong homology between the presumed nimQ polypeptide and budding yeast MCM2 (37) and Nda1 of fission yeast (58). NIMQ showed 58.2% identity and 73.5% similarity to MCM2 (Fig. 1B), and similar resemblance to Nda1 and also to related proteins in Drosophila (59) and human (60) (data not shown). MCM2 and its relatives encode the universal six-member MCM (minichromosome maintenance) family of proteins that form a complex near origins of replication and are required for DNA synthesis. The MCM proteins are required for initiation of DNA synthesis and have been implicated as components of the licensing factor system that limits DNA replication to once per cell cycle (39–41, 61).

Sequence identity among NIMQ, Mem2, and Nda1 spans most of the coding region except the N and C termini. Although the frequency of identical amino acids in the N-terminal —175 amino acids is relatively low, the distribution of charged residues is well conserved. Each protein begins with a short basic region, followed in order by a region rich in proline and serine, a highly acidic domain —25 residues long, and then a region of about 100 amino acids with a high proportion of acidic and basic residues. The novel CX4CX3CX2CX1C postulated zinc finger common to Mem2 and relatives in other species is also preserved in NIMQ (Fig. 1B).

Figure 1

G1/S nimQ20-arrested Cells Are Deficient in Tyr-15 Phosphorylation of p34CDC2—We have shown previously that inhibition of DNA replication by HU increases the level of Tyr-15 phosphorylated p34cdc2 (13). This increase in Tyr-15 phosphorylated p34cdc2 occurs both at 32°C and 42°C after addition of HU to wild type cells (Fig. 2A). It also occurs if DNA replication is inhibited by temperature-sensitive inactivation of nimP (Fig. 2C, nimP19 and nimP22), which encodes DNA polymerase ε.2 However, this response is lacking after inhibition of DNA replication by inactivation of nimQ(MCM2 or when HU is added after nimQ20 arrest (Fig. 2A, nimQ20).

Lack of p34cdc2 Tyr-15 phosphorylation is not due to an inability of p34cdc2 to bind to NIMQε because a com-

2 G. E. Crawford, A. N. Wexler, and S. W. James, unpublished data.
A parable amount of p34\(^{cd2}\) is co-immunoprecipitated with NIME\(^{cd2}\) in cells with or without nimQ\(^{MCM2}\) function (Fig. 2B). In addition, this defect is not simply caused by lower levels of NIME\(^{cd2}\) in cells lacking NIME\(^{cd2}\), as high levels of NIME cyclin B accumulate in the nimQ20MCM2 double mutant at 42 °C, but p34\(^{cd2}\) still remains Tyr-15 dephosphorylated (Fig. 2E) and consequently helps promote premature mitosis from G1/S without DNA replication (Figs. 3 and 4C).

Tyr-15 phosphorylation of p34\(^{cd2}\) occurs after DNA damage caused by MMS (56). However, this response is defective in cells first arrested without functional nimQ (Fig. 2D). nimQ is not an essential component of the system that Tyr-15 phosphorylates p34\(^{cd2}\) (an essential activator of Wee1 for example) because cells arrested in mitosis due to lack of BIME APC1 increase the level of Tyr-15-phosphorylated p34\(^{cd2}\) in response to DNA damage equally well with, or without, the function of NIME\(^{MCM2}\) (Fig. 2E).

Mitosis Is Partially Uncoupled from DNA Replication by the nimQ20MCM2 Mutation—Previous studies failed to reveal a mitotic phenotype associated with the nimQ20 mutation (35, 48). However, germination in complex media, paying particular attention to the DNA replication status in cells lacking NIME or Mcm2, showed significant differences. Cells lacking NimQ are unable to synthesize DNA and homology between NimQ and Mcm2. A, spores of SWJ186 (nimQ20, choA1) were germinated at permissive (30 °C) and restrictive (43 °C) temperatures, and the synthesis of DNA was monitored by incorporation of [\(3H\)]adenine into DNA. B, incorporation of [\(3H\)]adenine at 30 °C; C, incorporation of [\(3H\)]adenine at 43 °C. B, comparison of the amino acid sequences of NIME and Mcm2 proteins. The NIME sequence (upper) and the Mcm2 sequence (lower) were aligned using the GAP program of the UWCGC package with the gap weight and length weight set at 3.0 and 0.1, respectively. The acidic region and zinc finger motif of each polypeptide are double underlined.
nimQ<sup>MCM2</sup> and Cell Cycle Progression

As the slightly elevated mitotic index seen for <i>nimQ<sup>20</sup> MCM2</i> was transient (Fig. 3A), it is possible that in the double <i>nimQ<sup>20</sup> MCM2</i> + <i>bimE<sup>APC1</sup></i> mutant lack of APC function caused by the <i>bimE<sup>APC1</sup></i> mutation (25, 29) is trapping cells at a mitotic stage after they have prematurely progressed through the cell cycle. <i>nimQ<sup>20</sup> MCM2</i> cells were therefore germinated at 42 °C in the presence of nocodazole to trap cells at the first mitosis. This treatment partially elevated the mitotic index observed for the <i>nimQ<sup>20</sup> MCM2</i> strain (Fig. 3B) but still the mitotic index fell well short of that observed in the <i>nimQ<sup>20</sup> MCM2</i> + <i>bimE<sup>APC1</sup></i> double mutant strain (Fig. 3A). Thus, inhibition of APC function appears to markedly enhance the premature mitosis phenotype observed when <i>nimQ<sup>20</sup> MCM2</i> function is impaired and not just trap cells in a mitotic state. In further support of this contention, it should be noted that lack of both <i>nimQ<sup>MCM2</sup></i> and <i>bimE<sup>APC1</sup></i> promotes mitosis significantly faster than lack of either individually (Fig. 3A).

Importantly, addition of 100 mM HU was able to prevent premature mitosis in the double <i>nimQ<sup>20</sup> MCM2</i> + <i>bimE<sup>APC1</sup></i> mutant strain if it was added at the permissive temperature to arrest cells at S phase prior to shift to the restrictive temperature. After S phase arrest caused by HU, inactivation of BIME<sup>APC1</sup>, or a combination defect in BIME<sup>APC1</sup> and NIMQ<sup>MCM2</sup>, had a limited capacity to promote mitosis (Fig. 3D). This is in marked contrast to the premature mitosis induced in these strains when HU was present at the same time that BIME<sup>APC1</sup> and NIMQ<sup>MCM2</sup> were inactivated, during which cells will arrest initially at the <i>nimQ<sup>20</sup></i> arrest point (Fig. 3C). This demonstrates that there is a difference in mitotic regulation when DNA replication is arrested by HU (checkpoint over mitotic initiation fully engaged) compared with arrest by absence of NIMQ<sup>MCM2</sup> (checkpoint not fully engaged). The major difference we have observed is tyrosine phosphorylation of p34<sup>cdc2</sup> at the HU arrest point but not at the <i>nimQ<sup>20</sup> MCM2</i> arrest point (Fig. 2, A and C).

As tyrosine phosphorylation of p34<sup>cdc2</sup> is regulated by the opposing activities of the Wee1 tyrosine kinase and Cdc25 tyrosine phosphatase (62), lack of tyrosine-phosphorylated p34<sup>cdc2</sup> in <i>nimQ<sup>20</sup></i>-arrested cells could be caused by elevated Cdc25 phosphatase activity or lack of Wee1 kinase activity. If the Wee1 kinase is inactive at the <i>nimQ<sup>20</sup></i> arrest point, leading to lack of tyrosine-phosphorylated p34<sup>cdc2</sup>, then inactivation of the Cdc25 phosphatase should not prevent entry into mitosis. However, if the Wee1 kinase is active at the <i>nimQ<sup>20</sup></i> arrest point, and p34<sup>cdc2</sup> is being dephosphorylated by Cdc25 to produce non-tyrosine-phosphorylated p34<sup>cdc2</sup>, then inactivation of Cdc25 may prevent entry into mitosis. In the latter scenario, inactivation of Cdc25 would allow Wee1 tyrosine-phosphorylated p34<sup>cdc2</sup> to accumulate, which would consequently prevent any premature mitosis in <i>nimQ<sup>20</sup></i>-arrested cells. When a <i>nimQ<sup>20</sup> + nimT<sup>23</sup> cdc25</i> double mutant strain was germinated at 42 °C, the number of cells that entered premature mitosis was markedly reduced as compared with those in the single <i>nimQ<sup>20</sup></i> mutant strain (Fig. 3E). This indicates that Wee1 is active at the <i>nimQ<sup>20</sup></i> arrest point but that <i>nimT<sup>23</sup> cdc25</i> has greater activity to promote dephosphorylation of p34<sup>cdc2</sup> and allow some entry into mitosis. These data suggest that lack of tyrosine-phosphorylated p34<sup>cdc2</sup> in <i>nimQ<sup>20</sup></i>-arrested cells is largely due to the balance between the Cdc25 phosphatase and the Wee1 kinase favoring dephosphorylation, perhaps due to attention to early time points, revealed a slightly elevated mitotic index caused by the single <i>nimQ<sup>20</sup> MCM2</i> mutation germinated at 42 °C and, as reported previously (35), a dramatic premature entry into mitosis in the <i>nimQ<sup>20</sup> MCM2</i> + <i>bimE<sup>APC1</sup></i> double mutant strain (Fig. 3A).
high Cdc25 phosphatase activity still being present, as it is known to be activated at the G₂/M transition.

Initiation of mitosis in *A. nidulans* requires activation of both p34\(^{cdcd2}\)/cyclin B H1 kinase and the NIMA kinase. To determine if NIMA kinase is also required for the premature mitosis in the *nimQ150*–arrested cells, we generated a *nimQ150 nimA5* double mutant strain and examined the ability of the double mutant cells to initiate premature mitosis when germinated at 42 °C. As shown in Fig. 3E, lack of NIMA function prevented premature mitosis in the *nimQ150*–arrested cells, demonstrating that NIMA kinase is required for the low level of premature mitosis at G₁/S seen in the *nimQ150*–arrested cells.

**Anaphase Can Occur without APC Function in the Absence of *nimQ150Cmc2***—DAPI staining of mitotic cells seen when *nimQ150Cmc2* conidia were germinated at 42 °C revealed that many (55%, Fig. 4B) had progressed into anaphase and contained segregated DNA masses, some of which were not equal in intensity (data not shown, but see Fig. 4A, C, and E). Addition of HU did not affect anaphase progression in this strain, but microtubule depolymerization utilizing nocodazole completely suppressed DNA segregation, indicating it to be mitotically driven (Fig. 4B). Progression into anaphase was similarly observed (60%) in the double *nimQ150Cmc2 nimT23* mutant strain with or without HU addition (Fig. 4A, C, and E; and B) but was totally absent in the single *nimT23* strain (Fig. 4A, A, and B; and B). The actual percentage of mitotic cells progressing into anaphase measured in these experiments is conservative as visualization of DNA segregation is dependent upon the orientation of DNA within individual cells. Inactivation of 

**Fig. 3.** Cells lacking *nimQ150Cmc2* have a limited ability to initiate mitosis but cells lacking both *nimQ150Cmc2* and *bime7Apc1* initiate precocious mitosis in the absence of DNA replication. A, chromosome mitotic index (%CMI) of *nimQ20*, *bime7*, and *nimQ20 + bime7* mutant cells germinated at 32 °C (the permissive temperature) and at 42 °C (the restrictive temperature). Samples were taken at the time points indicated, fixed in glutaraldehyde, and stained with DAPI. CMI represents percentage of cells containing condensed mitotic chromatin. As the *bime7* single and the *nimQ20 + bime7* double mutant cells had very similar chromosome mitotic indexes when germinated at 32 °C, the chromosome mitotic index for the *bime7* cells is shown only. B, chromosome mitotic index of a wild type and the *nimQ20* mutant strains germinated at 42 °C in the presence and absence of 5 µg/ml nocodazole. Nocodazole was added to trap cells in mitosis. C, a time course of chromosome mitotic index (%CMI) of *nimQ20*, *bime7*, and *nimQ20 + bime7* mutant cells germinated at 42 °C in the presence of 100 mM HU. D, chromosome mitotic index (%CMI) of *bime7* and *nimQ20 + bime7* mutant cells, germinated at 32 °C for 7 h and then treated with or without HU at 32 °C for 2 h before temperature shift to 42 °C to inactivate NIMQ150Cmc2 and BIMEAPC1. Note the synergy between lack of NIMQ150Cmc2 and lack of BIMEAPC1 in promoting mitosis when HU was added to germinating cells at 42 °C (C, ● *nimQ20 + bime7 + HU*) but the lack of synergy when HU was added first to cells at 32 °C to arrest S phase before shifting to 42 °C to inactivate NIMQ150Cmc2 and BIMEAPC1 (D, ● *nimQ20 + bime7*). E, chromosome mitotic index (%CMI) of *nimQ20*, *nimQ20 + nimT23* and *nimQ20 + nimA5* double mutant cells germinated at 42 °C.
n imQ^{MCM2} and Cell Cycle Progression

Absence of NIMQ^{MCM2} function could promote anaphase in the double nimQ^{MCM2} + bimE7 APC1 mutant strain by restoring APC function. Alternatively, absence of NIMQ^{MCM2} could bypass the requirement for the APC to promote anaphase. We therefore assayed p34^{cdc2} H1 kinase activity and NIME cyclin B levels in bimE7 APC1, nimQ^{MCM2}, and double mutant strains germinated for 6 h at 42 °C (Fig. 4C). Levels of NIME cyclin B and H1 kinase activity were equally elevated in both the bimE7 APC1 and nimQ^{MCM2} + bimE7 APC1 strains. This suggests that lack of nimQ^{MCM2} function when bimE7 APC1 is inactivated does not allow activation of the APC, because NIME cyclin B levels are elevated and cells are arrested in mitosis. Instead, the data indicate that lack of nimQ^{MCM2} bypasses the requirement for the APC to promote anaphase.

Anaphase progression without APC function, as seen in the double nimQ^{MCM2} + bimE7 APC1 strain, is most likely due to the absence of sister chromatids as without sister chromatid cohesion a reductional anaphase has previously been observed in S. cerevisiae cdc6 mutants that fail to enter S phase but go into mitosis (63) and also occurs in a cdc6 mutant lacking APC function (64).

The SPB Duplication Cycle Is Uncoupled from the DNA Replication Cycle in nimQ20-arrested Cells—S phase arrest caused by the nimQ20^{MCM2} mutation was also remarkable because this arrest allowed uncontrolled duplication of spindle pole bodies. Arrest in S phase or G2 normally completely prevents spindle formation and SPB re-duplication (Fig. 5). For example, conidia of nimP22, nimA5, and nimT23 containing strains germinated at 42 °C totally prevents spindle formation as judged by IIF using α-tubulin staining and limits SPB duplication to one round of replication (Fig. 5, shown for nimP22POL in Fig. 6). Under the same conditions, 10% of the nimQ20^{MCM2} cells displayed mitotic spindles (Fig. 5A), many of which contained multiple spindle pole bodies giving rise to triangular spindles, splayed spindles, and other spindle morphological novelties (Fig. 6, nimQ20 and data not shown). An additional 20% of these cells had other less clearly defined abnormalities typified by thick microtubule bundles (data not shown).

Using indirect immunofluorescence to visualize γ-tubulin, the number of SPBs (57, 65) in the nimQ20^{MCM2} strain was significantly greater after germination at 42 °C than for the S and G2 arresting nim mutants (Fig. 5B). In addition, the number of SPBs in the nimQ20^{MCM2} strain was higher than in a wild type strain. For the non-mutant strain, the average number of SPBs per cell was 1.81, but for the nimQ20^{MCM2} strain, each cell had on average 2.78 SPBs after 6 h germination at 42 °C (Fig. 5B). This suggests not only that the SPB cycle is uncoupled from the DNA replication cycle in the nimQ20^{MCM2}-arrested cells but that the SPB replication cycle proceeds significantly faster than normal.

DISCUSSION

The nimQ20^{MCM2} mutation joins a group of mutations that interfere with temporal control of S phase and mitosis, which

HU or nocodazole on DNA segregation, the nimQ20 and the nimQ20 + bimE7 mutant cells were also germinated in the presence of 100 mM HU and 5 μg/ml nocodazole, respectively. C, p34^{cdc2} H1 kinase and levels of NIME cyclin B in bimE7, nimQ20, and nimQ20 + bimE7 mutant cells 6 h after germination at 32 °C or at 42 °C. After immunoprecipitation with NIME cyclin B-specific antiserum, the p34^{cdc2} H1 kinase activity was assayed using histone H1 as substrate in the presence of [γ-32P]ATP and was detected by autoradiography. NIME cyclin B was detected by ECL with NIME cyclin B-specific antibodies (E-8). After NIME cyclin B detection, the blot was stripped and then detected for NIMX^{cdc2} by ECL with NIMX^{cdc2} (E-77)-specific antibodies.

FIG. 4. Cells lacking NIMQ^{MCM2} can initiate anaphase in the absence of APC function. A, micrographs showing DAPI stained bimE7 cells germinated in the absence (a) or presence (b) of 100 mM HU, and DAPI-stained nimQ20 + bimE7 double mutant cells germinated in the absence (c) or presence (e) of 100 mM HU, for 6 h at 42 °C. d is a phase micrograph corresponding to c, and f corresponds to e. Arrows indicate segregated DNA masses within a single cell. Bar is approximately 2 μm. B, DNA segregation in mitotic cells lacking NIMQ^{MCM2}. Conidiospores derived from nimQ20, bimE7, and nimQ20 + bimE7 mutant strains were germinated at 42 °C for 6 h, and then fixed and stained with DAPI. Percentages of mitotic cells with segregated DNA were then determined by fluorescence microscopy. To see the effect of nimQ^{MCM2} therefore allows anaphase progression in the apparent absence of APC function.

Absence of NIMQ^{MCM2} function could promote anaphase in the double nimQ^{MCM2} + bimE7 APC1 mutant strain by restoring APC function. Alternatively, absence of NIMQ^{MCM2} could bypass the requirement for the APC to promote anaphase. We therefore assayed p34^{cdc2} H1 kinase activity and NIME cyclin B levels in bimE7 APC1, nimQ^{MCM2}, and double mutant strains germinated for 6 h at 42 °C (Fig. 4C). Levels of NIME cyclin B and H1 kinase activity were equally elevated in both the bimE7 APC1 and nimQ^{MCM2} + bimE7 APC1 strains. This suggests that lack of nimQ^{MCM2} function when bimE7 APC1 is inactivated does not allow activation of the APC, because NIME cyclin B levels are elevated and cells are arrested in mitosis. Instead, the data indicate that lack of nimQ^{MCM2} bypasses the requirement for the APC to promote anaphase.

Anaphase progression without APC function, as seen in the double nimQ^{MCM2} + bimE7 APC1 strain, is most likely due to the absence of sister chromatids as without sister chromatid cohesion a reductional anaphase has previously been observed in S. cerevisiae cdc6 mutants that fail to enter S phase but go into mitosis (63) and also occurs in a cdc6 mutant lacking APC function (64).

The SPB Duplication Cycle Is Uncoupled from the DNA Replication Cycle in nimQ20-arrested Cells—S phase arrest caused by the nimQ^{MCM2} mutation was also remarkable because this arrest allowed uncontrolled duplication of spindle pole bodies. Arrest in S phase or G2 normally completely prevents spindle formation and SPB re-duplication (Fig. 5). For example, conidia of nimP22, nimA5, and nimT23 containing strains germinated at 42 °C totally prevents spindle formation as judged by IIF using α-tubulin staining and limits SPB duplication to one round of replication (Fig. 5, shown for nimP22POL in Fig. 6). Under the same conditions, 10% of the nimQ^{MCM2} cells displayed mitotic spindles (Fig. 5A), many of which contained multiple spindle pole bodies giving rise to triangular spindles, splayed spindles, and other spindle morphological novelties (Fig. 6, nimQ20 and data not shown). An additional 20% of these cells had other less clearly defined abnormalities typified by thick microtubule bundles (data not shown).

Using indirect immunofluorescence to visualize γ-tubulin, the number of SPBs (57, 65) in the nimQ^{MCM2} strain was significantly greater after germination at 42 °C than for the S and G2 arresting nim mutants (Fig. 5B). In addition, the number of SPBs in the nimQ^{MCM2} strain was higher than in a wild type strain. For the non-mutant strain, the average number of SPBs per cell was 1.81, but for the nimQ^{MCM2} strain, each cell had on average 2.78 SPBs after 6 h germination at 42 °C (Fig. 5B). This suggests not only that the SPB cycle is uncoupled from the DNA replication cycle in the nimQ^{MCM2}-arrested cells but that the SPB replication cycle proceeds significantly faster than normal.

DISCUSSION

The nimQ^{MCM2} mutation joins a group of mutations that interfere with temporal control of S phase and mitosis, which

HU or nocodazole on DNA segregation, the nimQ20 and the nimQ20 + bimE7 mutant cells were also germinated in the presence of 100 mM HU and 5 μg/ml nocodazole, respectively. C, p34^{cdc2} H1 kinase and levels of NIME cyclin B in bimE7, nimQ20, and nimQ20 + bimE7 mutant cells 6 h after germination at 32 °C or at 42 °C. After immunoprecipitation with NIME cyclin B-specific antiserum, the p34^{cdc2} H1 kinase activity was assayed using histone H1 as substrate in the presence of [γ-32P]ATP and was detected by autoradiography. NIME cyclin B was detected by ECL following Western blotting of 100 μg total protein extract using anti-NIME cyclin B-specific antibodies (E-8). After NIME cyclin B detection, the blot was stripped and then detected for NIMX^{cdc2} by ECL with NIMX^{cdc2} (E-77)-specific antibodies.
also play a role in initiation of DNA replication (63, 66–71). These observations have led to the proposal that the act of initiating DNA replication activates a negative control system preventing initiation of mitosis during S phase (69, 72).

The nature of this negative control system has not been demonstrated previously. Here we demonstrate that upon execution of \( \text{nimQ}^{20} \) function Tyr-15 phosphorylation of \( p34^{\text{cdc}2} \) becomes activated to help prevent mitotic initiation during S phase. Thus, lack of \( \text{nimQ} \) not only prevents DNA replication, it also prevents Tyr-15 phosphorylation of \( p34^{\text{cdc}2} \).

This result provides a molecular explanation for why mitosis can be initiated before DNA replication in the double \( \text{nimQ}^{20} \) mutant strain of \( A. \text{nidulans} \) (35). We have recently demonstrated that arrest of mitotic initiation after S phase in \( A. \text{nidulans} \) is mediated by the combined actions of \( \text{nimP} \) and Tyr-15 phosphorylation of \( p34^{\text{cdc}2} \). By contrast, S phase arrest caused by mutation of the \( \text{nimP} \) gene or by HU (at which initiation complexes would be activated but immediately stalled due to lack of deoxynucleotides) allows Tyr-15 phosphorylation of \( p34^{\text{cdc}2} \), and after the HU arrest point, there is no longer a requirement for \( \text{nimQ} \) in the Tyr-15 phosphorylation of \( p34^{\text{cdc}2} \). The function of \( \text{nimQ}^{20} \) therefore acts like a trigger to allow Tyr-15 phosphorylation of \( p34^{\text{cdc}2} \) upon initiation of DNA replication, after which it is not required for Tyr-15 phosphorylation of \( p34^{\text{cdc}2} \).

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at a stage that blocks activation of Tyr-15 phosphorylation of p34<sup>cdc2</sup> (inactivate nimQ<sup>MCM2</sup>) and also inactivating APC function (bimE<sup>APC1</sup> mutation), negative controls over MPF, and other mitotic regulators such as NIMA (13), are removed and mitosis is prematurely initiated from G<sub>1</sub>/S.

Coordination of APC and Tyr-15 Phosphorylation of p34<sup>cdc2</sup>-At some point during progression out of mitosis into interphase, the APC needs to be inactivated (it is activated to permit exit from mitosis) to allow mitotic cyclin accumulation for the next mitosis. Additionally, Tyr-15 phosphorylation of p34<sup>cdc2</sup> needs to be turned back on (it is turned off after activation of Cdc25 at G<sub>2</sub>/M) to prevent premature activation of pre-MPF during interphase. We show that Tyr-15 phosphorylation of p34<sup>cdc2</sup> during G<sub>2</sub>/S progression occurs after the function of nimQ<sup>MCM2</sup>, which may involve down-regulation of Cdc25, which is activated during G<sub>2</sub>/M of the previous cycle. Because APC activity is known to remain active during G<sub>1</sub> in both yeast (73) and human cells (74), it is likely that cyclin B cannot normally accumulate and bind p34<sup>cdc2</sup> in G<sub>1</sub> of many cell types and so they have no need to negatively regulate p34<sup>cdc2</sup> by Tyr-15 phosphorylation during this cell cycle stage. However, as soon as APC activity is reduced during the G<sub>1</sub>/S transition, cyclin B begins to accumulate in preparation for the next mitosis, it becomes critical to prevent production of MPF, which is achieved by allowing Tyr-15 phosphorylation of p34<sup>cdc2</sup>. A transition thus occurs, during which negative control over mitotic p34<sup>cdc2</sup> is transferred from the APC in G<sub>1</sub> to include the Tyr-15 phosphorylation of p34<sup>cdc2</sup> in S phase. Here we show that this transition is dependent upon nimQ<sup>MCM2</sup> function.

The requirement for nimQ<sup>MCM2</sup> to trigger Tyr-15 phosphorylation of p34<sup>cdc2</sup> at G<sub>1</sub>/S is likely to involve its capacity to promote the initiation of DNA replication as nimQ<sup>MCM2</sup> is not required for Tyr-15 phosphorylation of p34<sup>cdc2</sup> after DNA replication has been initiated (for instance, at the HU-arrest point). Additionally, as mentioned before, nimQ20 is not unique as numerous mutations have been identified that interfere with temporal control of S phase and mitosis, which also play a role in the initiation of DNA replication (63, 66–71).

Tightly coupling Tyr-15 phosphorylation of p34<sup>cdc2</sup> to initiation of DNA replication ensures that mitosis is not promoted prematurely during S phase. We further propose that APC inactivation may occur only after Tyr-15 phosphorylation of p34<sup>cdc2</sup> is activated to ensure that mitosis is not initiated in G<sub>1</sub> by accumulation of cyclin B (Fig. 7).

Work using Schizosaccharomyces pombe has also indicated the existence of another level of control over mitotic p34<sup>cdc2</sup> during G<sub>1</sub> because p34<sup>cdc2</sup> is tyrosine-dephosphorylated during M/G<sub>1</sub> until execution of Start (75). This pre-Start checkpoint was suggested to act in an undefined manner by preventing formation of the p34<sup>cdc2</sup>-p56<sup>cdc13</sup> complex. Our studies partially support such a mechanism but, importantly, more directly implicate the APC in the pre-Start, or pre-DNA replication, negative control of p34<sup>cdc2</sup>. It is worth pointing out that at the nimQ20 arrest point some cyclin B protein is present and significant H1 kinase activity exists (Fig. 4C). This likely helps explain why some nimQ20-arrested cells enter a mitotic state from G<sub>1</sub>/S. However, only upon inactivation of the APC by the bimE<sup>APC1</sup> mutation do nimQ20-arrested cells increase cyclin B protein levels and H1 kinase activity and so overwhelmingly rapidly enter mitosis (Fig. 4C). The possibility exists that BIME<sup>APC1</sup> also negatively regulates the NIMA kinase as NIMA is also required to promote premature mitosis in the nimQ20-arrested cells and it is unstable during exit from mitosis (76). We have shown previously that tyrosine-dephosphorylated p34<sup>cdc2</sup> in combination with lack of BIME function leads to deregulation and activation of NIMA in S phase, a point in the cell cycle when NIMA is normally inactive (13).

A Role for nimQ<sup>MCM2</sup> in Spindle Pole Body Duplication—The DNA licensing hypothesis proposed that DNA licensing factors, being essential for initiation of DNA replication, were irreversibly changed upon initiation of DNA replication and could not be replenished to promote DNA replication again until passage through mitosis (38). Based upon movement of MCM proteins in and out of the yeast nucleus during the cell cycle (71–79) and MCM protein association with DNA and phosphorylation state during the cell cycle (60, 71, 80–84), this family of proteins (including Mgm2) have been suggested as candidates for licensing factor. Purification of licensing factor from Xenopus egg extracts identified two complexes having licensing activity (85, 86). One of these (RFL-M) contains MCM homologues, further supporting the role of this class of protein in the regulation of DNA replication to one round per cell cycle. Our data additionally suggest a potential role for nimQ<sup>MCM2</sup> in the regulation of SPB duplication. Inactivation of nimQ<sup>MCM2</sup> prevents DNA replication, which normally prevents SPB duplication (Fig. 5B). However, SPB duplication is uncoupled from these normal controls in the nimQ<sup>20MCM2</sup>-arrested cells, leading to the production of multiple SPBs in the absence of DNA replication.
The elevated numbers of SPBs generated when nimQMC2 is inactivated were functional because they were capable of nucleating microtubules leading to the generation of tripolar and other multipolar spindles. Indeed, at the resolution of the light microscope, many of the excess SPBs observed were similar in size to normal SPBs. A similar phenotype has been reported in mouse embryonic fibroblasts lacking the p53 tumor suppressor protein. Cells containing multiple centrosomes forming tripolar and other abnormal spindles were observed in cultured mouse cells lacking p53 (87). The defects observed in mouse cells lacking p53 are remarkably similar, in relation to microtubule organizing centers, to what we have observed for A. nidulans cells lacking the function of an Mcm2 homologue. The potential relationship between the roles of p53 and Mcm2 in microtubule organizing center duplication will be of interest in higher eukaryotic cells.

It is intriguing that a function known to be involved in the regulation of DNA replication may also play a key role in the regulation of SPB duplication. Perhaps these two levels of regulation are linked in some way via the Mcm2 function to ensure normal coupling of the DNA replication cycle and the SPB duplication cycles. However, in the present study it is not certain how Mcm2, in addition to its role in initiation of DNA replication, regulates SPB duplication. Regardless of what potential roles Mcm2 may play in the regulation of SPB duplication, the uncoupling of the SPB from the chromosomal cycle in cells lacking Mcm2 could help us understand how these two cycles are normally coordinated during the cell cycle.

Conclusions—We present a model whereby control of mitosis during the G1 to S transition is transferred from the APC in G1 to include the Tyr-15 phosphorylation of p34cdc2 after initiation of DNA replication (Fig. 7). This transition from APC-controlled inhibition of mitosis to inhibition by Tyr-15 phosphorylation of p34cdc2 was revealed through examination of a mutation in the nimQMC2 gene, which is required for initiation of DNA synthesis. We also show that the SPB duplication cycle is uncoupled from the chromosomal cycle in cells lacking nimQMC2, suggesting a potential role for Mcm2 in the control of SPB duplication. The data provide a molecular logic for negative control of mitosis during G1/S, which is likely to be applicable to other eukaryotes that regulate mitotic exit via the APC and mitotic entry by Tyr-15 dephosphorylation of p34cdc2.

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