Phosphorylation of the Mitotic Regulator Protein Hec1 by Nek2 Kinase Is Essential for Faithful Chromosome Segregation*

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Hec1 (highly expressed in cancer) plays essential roles in chromosome segregation by interacting through its coiled-coil domains with several proteins that modulate the G2/M phase. Hec1 localizes to kinetochores, and its inactivation either by genetic deletion or antibody neutralization leads to severe and lethal chromosomal segregation errors, indicating that Hec1 plays a critical role in chromosome segregation. The mechanisms by which Hec1 is regulated, however, are not known. Here we show that human Hec1 is a serine phosphoprotein and that it binds specifically to the mitotic regulatory kinase Nek2 during G2/M. Nek2 phosphorylates Hec1 on serine residue 165, both in vitro and in vivo. Yeast cells are viable without scNek2/Kin3, a close structural homolog of Nek2 that binds to both human and yeast Hec1. When the same Ser residue is mutated to Ala or yeast Hec1 mutant changing Ser165 to Ala does not. Mutations changing the same Ser residues to Glu, to mimic the negative charge on its dynamic expression and subcellular localization in cytosol, nucleus, and chromosome portions other than the centromesome (21, 22). Nek2 may therefore have several functions in regulating cell proliferation, not only a role in G2/M phase progression. In budding yeast, a structural homolog of Nima and Nek2, scNek2/Kin3 (also known as Fun52 and Npk1), has been identified (23–25), but its functional similarity to human Nek2 has not yet been established.

In this report, we demonstrate how the function of Hec1 is regulated during G2/M phases by characterizing the previously reported interaction between Hec1 and Nek2 (5). We take advantage of Nek2 and Hec1 homologs, specific point mutants, and the ability to assay chromosomal segregation errors in budding yeast to show that Hec1 is phosphorylated by Nek2.

Mitosis must be precisely regulated and checked for faithful partitioning of chromosomes during a short but crucial period of the cell division cycle. Since the basic mechanics of chromosome segregation and checkpoint control are conserved in all eukaryotes, yeast and other fungi are excellent tools for dissecting mechanisms that apply to mammalian cell cycle proteins with clear homologs in lower eukaryotes. The novel coiled-coil protein Hec1 plays important roles in chromosome condensation and cohesion by interacting with structural components of the mitotic chromosome, including SMC1 (structure of the mitotic chromosome 1) complexes (1–3) and the kinetochore protein Ctf19p (4). Hec1 also regulates S protein (activity through interaction with SMC1/CIM5/subunit 7, p45/Trip1 (Sug/LCIM3/subunit 8), and p44.5 (subunit 9) (5). These Hec1-interacting proteins were first identified in yeast two-hybrid assays using the coiled-coil region of Hec1 as bait (1). The mechanisms by which Hec1 itself is regulated during G2 and M phases are not yet known, however. Since Hec1 has a structural and functional homolog in S. cerevisiae (scHec1, also known as TID3, NDC80, and YIO4) (3, 6), the consequences of its interactions with other proteins can be meaningfully explored in yeast.

Protein kinases have been shown to play important roles regulating G2/M phase progression. One such kinase, NimA (never in mitosis Δ), which phosphorylates specific proteins on serine and threonine residues, is vital in Aspergillus nidulans for entry into mitosis (7–9). Cells harboring temperature-sensitive mutations of nimA arrest specifically in G2 at nonpermissive temperature, but rapidly and synchronously enter mitosis upon shift to permissive temperature (10). The expression of NimA is tightly regulated during the nuclear division cycle, peaking in G2 and M phases. NimA, like Hec1, has also been shown to influence faithful chromosome segregation (7–9).

Kinesins with structural and functional homology to NimA exist in vertebrate cells (11–18). These NimA-related kinesins, or Neks, are purported to complement or function in manners similar to those of Cdc2 and other G2/M phase cyclin-dependent kinesins. Nek2, the homolog in human cells with the greatest structural similarity to NimA within the catalytic domain (19), is regulated in yeast in a manner similar to regulation of NimA in A. nidulans; its expression and serine/threonine kinase activity are highest during late G2 phase, when Nek2 is expected to function critically (7, 17, 20). Furthermore, a portion of Nek2 localizes to centrosomes and appears in mammalian cells to play a role similar to NimA in controlling entry into mitosis (17, 21). Nek2 may have more diverse roles during several phases of the cell cycle, from S phase to multiple phases of mitosis, based on its dynamic expression and subcellular localization in cytosol, nucleus, and chromosome portions other than the centromesome (21, 22). Nek2 may therefore have several functions in regulating cell proliferation, not only a role in G2/M phase progression. In budding yeast, a structural homolog of NimA and Nek2, scNek2/Kin3 (also known as Fun52 and Npk1), has been identified (23–25), but its functional similarity to human Nek2 has not yet been established.

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kinase during G2 and M phases. This specific modification is vital for Hec1 to coordinate faithful chromosome segregation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Synchronization**—Human bladder carcinoma T24 cells (American Type Tissue Collection, Manassas, VA) grown in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum were synchronized at G1 by density arrest and then released at time zero by replating in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum at a density of 2 × 10^5 cells/10 cm plate. At various time points thereafter (18 h for G1/S, 22 h for S, 32 h for G2), the cells were harvested. To obtain a cell population enriched in M phase, nocodazole (0.4 μg/ml) was added to the culture medium for 8 h prior to harvest (26).

**Yeast Strains, Reagents, and Media**—Yeast strains are described in Table I. Strains used in this study were grown in complete medium (YPD; 1% yeast extract, 2% peptone, and 2% dextrose) or in supplemented minimal medium with appropriate amino acids missing. The chemicals and medium components were purchased from Sigma and BD Industries (Franklin Lakes, NJ).

**Immunoprecipitation and Western Blot Analysis**—T24 cells resuspended in ice-cold Lysis 250 buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Nonidet P-40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride) were subjected to three freeze/thaw cycles (liquid nitrogen/37 °C) and then centrifuged at 14,000 rpm for 2 min at room temperature. The supernatants were used for immunoprecipitation as described (27). Briefly, anti-Hec1 antibody mAb1 9G3 (1 μg) or mouse polyclonal anti-Nek2 antisera (1 μl) was added to each supernatant. After a 1-h incubation, protein A-Sepharose beads were added, and incubation continued for another 1 h. Beads were collected, washed five times with lysis buffer containing hypertonic NaCl, and then boiled in SDS-loading buffer for immunoblot analysis as described (27). For the double immunoprecipitation experiment, Hec1 was immunoprecipitated from 8×-labeled T24 cells as above. The washed immunocomplex was then incubated at 100 °C for 5 min in 200 μl of disassociation buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1% SDS, and 5 μl dithiothreitol). The heated immunocomplex was then diluted with 1 ml of cold Lysis

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1 The abbreviations used are: mAb, monoclonal antibody; aa, amino acids; GST, glutathione-S-transferase.
Fig. 1. Cell cycle-dependent serine phosphorylation of Hec1. A, T24 cells were labeled with either [35S]methionine or [32P]orthophosphate and lysed. Lysates were immunoprecipitated with polyclonal anti-Hec1 antibodies (lanes 2 and 3), preimmune sera (lanes 1 and 4), or monoclonal anti-Hec1 antibodies (mAb 9G3) (lane 6). Lane 3 was performed as a double immunoprecipitation to eliminate nonspecific and co-immunoprecipitating proteins. The arrow indicates migration of the 76-kDa Hec1 protein. B, phosphoamino acid analysis of [32P]-labeled Hec1. The radioactively labeled protein was isolated and subjected to amino acid hydrolysis. The lysates were analyzed by thin layer chromatography using phosphorilated serine, threonine, and tyrosine as standards. Hec1 is primarily phosphorylated on serine residues. P, unincorporated, labeled phosphate; Ori, original spot. C, cell cycle-dependent phosphorylation of Hec1. T24 cells released from density arrest at G1 (lane 3) were labeled with [32P]-orthophosphate, lysed, and immunoprecipitated with mAb 9G3 at time periods corresponding to different phases of the cell cycle. Expression of Hec1 was detected by Western blotting with mAb 9G3, shown in the middle panel (G11, 11 h after release for G1; G18, 18 h after release for G/S; G24, S phase; G2, 32 h for G2/M phase). Phosphorylation of Hec1 (hsHec1p) is evident starting at S phase (lane 6, bottom panel) and becomes most prominent during M phase (lane 7, bottom panel). The phosphorylation pattern of p110m was used to mark cell cycle progression (top panel), as previously described (27).

300 mM NaCl. The fractions from the SP Sepharose column were loaded onto a nickel-Sepharose column (Amersham Biosciences) and eluted with 60 mM imidazole. The Hec1 protein was fractionated by Sephadex G-200 and immunoprecipitated with polyclonal anti-Hec1 serum, monoclonal anti-Hec1 antibodies (mAb 9G3), or preimmune sera (lanes 1, 2). Lane 5 was immunoprecipitated at G2 and M phases (lanes 5). Lane 3 was immunoprecipitated with polyclonal anti-Hec1 serum, monoclonal anti-Hec1 antibodies (mAb 9G3), or preimmune sera (lanes 1, 2). Lane 5 was immunoprecipitated at G2 and M phases (lanes 5).

Fig. 2. Interaction between Nek2 and Hec1 by GST pull-down assay. A, Sepharose beads bound with purified GST (lane 2) and GST fusions of Hec1 containing amino acids 56–642 (lane 3) or 251–618 (lane 4) were mixed with in vitro translated, [35S]methionine-labeled Nek2 (lane 1) and then washed extensively. The binding complexes were separated by SDS-PAGE, dried, and visualized by autoradiography. B, specific regions of Hecl bind to Nek2 by yeast two-hybrid assay. Deletion mutants containing the different coiled-coil domains of Hec1 were fused in frame to a GAL4 DNA binding domain. Nek2 was expressed as a GAL4 transactivation domain fusion. Yeast transformants with these two hybrid proteins were grown in liquid cultures and used for O-nitrophenyl-β-galactopyranosidase quantification of β-galactosidase activity. The -fold increase in activity compared with the host yeast strain Y150 is indicated. Assays were performed in triplicate for each transformation. C, cell cycle-dependent interaction between Hecl and Nek2. T24 bladder carcinoma cells were first density-arrested at G1 (lanes 2 and 3) and then released for reentry into the cell cycle. At different time points after release from density arrest (indicated above the lanes), cells were collected and lysed. The clarified lysates were immunoprecipitated with mAb 9G3 anti-Hec1 monoclonal antibodies (upper two panels) or anti-Nek2 antisera (lower two panels). Hecl and Nek2 co-immunoprecipitated at G2 and M phases (lanes 5 and 6).

For the anti-scNek2/Kin3 antibody, cDNA encoding full-length scNek2/Kin3 was fused to GST in frame; the fusion protein was purified and used as an antigen. Anti-Hecl and anti-scHecl antibodies have been described (1, 5). For anti-phosphorylated Hecl antibody, a synthetic phosphopeptide (A439; Fig. 3A) was coupled to keyhole limpet hemocyanin (KLH) and used as antigen.

RESULTS

HEC1 Is a Serine Phosphoprotein, and Its Phosphorylation Is Cell Cycle-dependent—To explore a potential mechanism by which Hecl is regulated, we tested whether Hec1 is modified by phosphorylation. T24 cells were labeled with either [35S]methionine or [32P]-orthophosphate and lysed. The lysates were immunoprecipitated with polyclonal anti-Hec1 serum, monoclonal anti-Hec1 antibodies (mAb 9G3), or preimmune serum and then separated by SDS-PAGE. The 76-kDa Hecl protein was recognized by both polyclonal and monoclonal 9G3 antibodies was labeled by [32P] (Fig. 1, lanes 5 and 6), showing Hecl C to be a phosphosulfate. Phosphoamino acid analysis showed Hecl C to be phosphorylated only on serine residues (Fig. 1B). To determine the cell cycle dependence of Hecl phosphorylation, T24 cells released from density arrest at G0 phase for different


different
Hec1 was detected by straight Western blotting with anti-A439 antibodies during cell cycle progression. T24 cells were phosphatase-treated Hec1.

**A.**

peptide sequence: LYPFALS$_{\text{Ser}}^\text{S}$KSSMYTV (A439)

**B.**

WB: mAb 9G3

α-A439

pHec1p

α-A439

**C.**

WB: mAb 9G3

CIP

T24-U

T24-M

α-A439

pHec1p

α-A439

Phosphorylation of Hec1 on Serine 165 in Vivo

We noted expression of both Hec1 and Nek2 was regulated during progression of the cell cycle (Fig. 2C). Co-immunoprecipitation of Nek2 and Hec1 occurred specifically during G$_2$ and M phases (Fig. 2C, lanes 5 and 6). The initiation of Hec1 phosphorylation (Fig. 2C, lane 6) corresponded to the same time period during which Nek2 was most abundant (Fig. 2C, lane 4), suggesting that Nek2 may phosphorylate Hec1 in vivo during G$_2$/M phase.

Phosphorylation of Hec1 on Serine 165 in Vivo

We noted that Hec1 has a potential phosphorylation site at serine 165 for both NimA and Nek2 (16, 20, 32) (Fig. 3A). To test whether Ser$^{165}$ of Hec1 is the authentic site phosphorylated by Nek2, an
Chromosome Segregation Requires Nek2 to Phosphorylate Hec1

antibody specifically recognizing a synthesized Hec1 phosphopeptide (Fig. 3A) was generated and used to examine the expression of phosphorylated Hec1 (Fig. 3B). Lysates from T24 cells, from cells synchronized at M phase, and from an unsynchronized population were immunoprecipitated with anti-Hec1 antibodies. The anti-A439 antibody recognized the phosphorylated form of Hec1 but did not recognize the unphosphorylated form from lysates treated with calf intestine phosphatase (Fig. 3B). In contrast, interaction between Hec1 and mAb 9G3 recognized both phosphorylated and unphosphorylated forms of Hec1 and was not affected by phosphatase treatment (Fig. 3B). The phosphorylated form of Hec1 was detected most abundantly by anti-439 in the lysates enriched for mitotic cells (Fig. 3C, lane 3). This finding is consistent with the ³²P labeling experiment shown in Fig. 1C, in which the phosphorylated form of Hec1 was most abundant at the G₂/M phase. Together, the results suggest that human Hec1 is phosphorylated on serine 165 in vivo.

Nek2 Phosphorylates Hec1 in Vitro—To determine whether Nek2 phosphorylates Hec1 directly, His-tagged, wild-type Hec1 and a specific human Hec1 mutant (hsHec1S165A) changing the putative Nek2 phosphorylation site at serine 165 into a neutral amino acid, alanine, were then expressed and purified to near homogeneity using a PET expression system (Fig. 4A). His-tagged Nek2 was expressed in a baculovirus system and purified by Ni⁺⁺-NTA affinity chromatography (Fig. 4B). Kinase reactions were then performed using purified Hec1 and hsHec1S165A mutant as substrates. Nek2 phosphorylated wild-type Hec1 (Fig. 4C, lane 3) but not hsHec1S165A (lane 5). Proteins immunoprecipitated by non-specific, preimmune antibodies (Fig. 4C, lane 1) or intentionally heat-inactivated Nek2 (Fig. 4C, lane 4) failed to phosphorylate Hec1. Furthermore, anti-A439 recognized the phosphorylated form of wild-type Hec1 (Fig. 4D, lane 2) but not the hsHec1S165A mutant even after the kinase reaction (Fig. 4D, lanes 3 and 4). Anti-A439 did not recognize the unphosphorylated form of wild-type Hec1 (Fig. 4D, lane 1). These results confirmed the residue on which Nek2 kinase phosphorylates human Hec1 is serine 165.

Yeast Kin3 Shares Similar Properties with Nek2—Human Hec1 (hsHec1) has a structural and functional homolog in yeast, scHec1/TID3/NDC80/YIO4, and is required for faithful chromosome segregation (3). Since Hec1 is specifically phosphorylated at the G₂ and M phases, phosphorylation of Hec1 by Nek2 may be critical for chromosome segregation. To address this possibility, a yeast model system was employed because well-established methods are available to assay chromosome segregation (2, 3, 28, 37). However, we first needed to identify a homolog of Nek2 in yeast that may phosphorylate scHec1. There is an open reading frame in the S. cerevisiae genome, Kin3/scNek2, which encodes a putative protein and could function as a serine/threonine kinase (23, 24). This protein shares relatively high homology (36.4% identity) with NimA and human Nek2 in the catalytic domain (Fig. 5A) and contains a coiled-coil domain in its C-terminal region that is similar to the same domains in the other two proteins (Fig. 5B). To test whether these C-terminal regions share similar abilities to physically interact with hsHec1p or scHec1p, Nek2p and scNek2p were synthesized in vitro for GST pull-down assays with both GST-hsHec1p and GST-scHec1p. Nek2p and scNek2p could bind both human and yeast Hec1p (Fig. 5C). These results suggested that scNek2 and Nek2 not only share homology at their N-terminal kinase domain sequences but that they also both have Hec1 binding activity at their C-terminal regions.

Changing glutamic acid 41 of NimA into glycine leads to a temperature-sensitive growth phenotype that arrests the cells in the G₂ phase at the nonpermissive temperature (8, 9). Interestingly, similar acidic residues have been found by other researchers to be highly conserved in the other kinases: residue
38 (glutamic acid) in Nek2 (19), and residue 55 (aspartic acid) in scNek2 (23, 24) (Fig. 5D). To test the functional similarity of these key regions among the kinases, glutamic acid 38 of Nek2 and aspartic acid 55 of scNek2 were each changed to glycine. Like the temperature-sensitive nima mutant (8, 9), the scNek2D55G mutant grew at 25 °C, arrested at 37 °C, and reentered the cell cycle when shifted back to the 25 °C (Fig. 6A). When the Nek2E38G mutant was introduced into scNek2 null cells, growth and propagation of the cells was temperature-sensitive as well (Fig. 6B). This temperature-sensitive phenotype was partially suppressed by expression of additional wild-type scNek2 or hsNek2 (Fig. 6C). Taken together, these results suggest that scNek2/Kin3 shares several similar functions with hsNek2 and that scNek2/Kin3 might function as an Nek2 homolog in S. cerevisiae.

Temperature-sensitive scNek2 Mutant Fails to Phosphorylate Hec1 at the Nonpermissive Temperature—To determine whether scNek2D55G may behave in a dominant negative fashion to arrest cells at nonpermissive temperature, we first generated specific antibodies and examined the physical interaction between scNek2p and scHec1p or scNek2p and hsHec1p, using co-immunoprecipitation (Fig. 7, A and B). In cells carrying the scNek2D55G mutant, the interaction between scNek2p and scHec1p or scNek2p and hsHec1p (Fig. 7, and D) was intact, as it was in wild-type scNek2 cells (Fig. 7, C and D). Moreover, the phosphorylation of hsHec1p on serine 165 was detected by anti-A439, both in wild-type and in scNek2D55G mutant cells at the permissive temperature, but not in scNek2D55G mutant cells at the nonpermissive temperature (Fig. 7E). These results suggest that phosphorylation of Hec1 by scNek2p is essential for cells to continue cycling, scNek2D55G thus appears indeed to be a dominant negative mutant; it can bind to Hec1 but cannot phosphorylate it.

Phosphorylation of hsHec1 Serine 165 Is Critical for Its Function in Chromosome Segregation—To examine whether the phosphorylation of human Hec1 (hsHec1) on serine 165 is important for hsHec1 to function, yeast strains containing specific hsHec1 mutations were created. The homolog of human Hec1 in S. cerevisiae (scHec1/TID3/NDC80/YIO4) has been characterized extensively and shown, like its mammalian counterpart, to be essential for chromosome segregation and yeast survival (3, 6). Furthermore, hsHec1 can complement the essential functions of scHec1 (3). Mutant constructs were created in which the critical serine residues phosphorylated by Nek2 in scHec1 (Ser201) and hsHec1 (Ser165) were mutated. scHec1S201A and hsHec1S165A substituted the neutral amino acid alanine for serine; scHec1S201E and hsHec1S165E substituted glutamic acid for serine to mimic the negative charge created by serine phosphorylation (Fig. 8A). To test whether these Hec1 mutants could complement scHec1 deficiency, they were introduced into the scHec1 null yeast strain. Both scHec1S201E and Hec1S165E were able to rescue yeast deficient in scHec1, but the scHec1S201A and hsHec1S165A mutants were not (Fig. 8B). These results suggest that phosphorylation of serine 165 (or serine 201 in yeast Hec1) is important for the function of Hec1.

To determine whether substituting glutamic acid for serine 165 in hsHec1 could rescue all essential functions of Hec1 in yeast, plating efficiency and chromosome segregation were examined in scHec1 null yeast rescued by either wild-type hsHec1 or hsHec1S165E. The plating efficiency of the scHec1Δ/hsHec1S165E strain was only 75% of the efficiency for the strain rescued by wild-type hsHec1 (Fig. 8C). This result suggested that the hsHec1S165E mutant was not fully functional in allowing faithful mitosis. To address this possibility, colony sectoring assays (2, 3, 28) were performed in the two yeast strains to monitor chromosome segregation. Yeast cells null for scHec1 and rescued by the hsHec1S165E were 10 times more prone to segregation errors, especially chromosome loss (1:0 events), compared with cells rescued by wild-type hsHec1 (Table II). Yeast cells lacking scNek2, although viable, were thought to have subtle errors in chromosome segregation. To test this hypothesis directly, scNek2 null cells were examined by colony sectoring assays. They were found to have 50-fold higher rates of errors of chromosomal losses (1:0 events) and 6-fold higher rates of nondisjunction (2:0 events) (Table II). Taken together, the results suggest that precisely regulated phosphorylation of Hec1 by Nek2 is critical for accurate chromosome segregation during mitosis.

**DISCUSSION**

In this paper, we have shown that Hec1 binds to Nek2 both in vitro and in vivo at G2/M phase. Nek2 specifically phosphorylates human Hec1 on serine residue 165 in a cell cycle-dependent manner, with a peak activity during G2/M. The phos-
Phosphorylation of Hec1 by Nek2 is critical for yeast survival. A. Hecl phosphorylation sites for Nek2. The potential Nek2 Ser phosphorylation sites in scHecl (Ser201) and hsHecl (Ser165) were mutated to Ala (S201A,S165A) or Glu (S201E,S165E) (37). B, both scHeclS201A and hsHeclS165A failed to rescue cells null for scHecl. Only wild-type scHecl or hsHecl and the scHeclS201E and hsHeclS165E mutants, in which glutamic acid substitution for serine mimics the negative charge created by serine phosphorylation, were able to rescue yeast deficient for scHecl. C, plating efficiency of yeast rescued by wild-type hsHecl or by hsHeclS165E. Two hundred cells from log phase cultures were plated onto solid plates. The surviving cells were scored for colonies formed on plates after 3 days in culture at 30 °C. The results are shown as means ± S.E. from three independent experiments.

The Role of Hec1 in Chromosome Segregation Is Highly Conserved—Hec1, a coiled-coil protein highly expressed in most cancer cells, is crucial for faithful chromosome segregation. Cells microinjected with anti-Hec1 antibodies undergo aberrant mitosis, with grossly inequitable distribution of chromosomes. Prevention of Hec1 phosphorylation can only partially rescue faithful chromosome segregation and subsequent viability of daughter cells.
Thus, phosphorylation of Hec1 must be tightly regulated and coordinated along with the cell cycle progression.

Hec1 Is a Substrate of Nek2—Specific phosphorylation during G2/M is required for Hec1 to function properly and for chromosome segregation to occur faithfully. Our studies have clearly shown Hec1 to be an authentic substrate of Nek2. Failure of Nek2 to phosphorylate Hec1 during G2/M leads to errors in segregation of chromosomes. Another potential substrate of Nek2, C-Nap1, localizes to centrioles in both mother and daughter cells and has coiled-coil structures appropriate for other protein-protein interactions (33). The structure of C-Nap1 might allow it to connect proximal ends of centrioles to each other, although this concept at present remains speculative (33). Nevertheless, it is interesting to note that both Nek2 substrates, C-Nap1 and Hec1, localize either to centrioles or kinetochores (1, 3, 6, 21). They are therefore positioned precisely at the mitotic apparatus, along with the machinery responsible for chromosome segregation.

Potential Redundant Kinases for Hec1—scNek2/kin3 has been purported to be the homolog of Nek2 and NimA because the three proteins have significant structural similarities. However, complete deletion of scNek2 had little influence on yeast survival and led to some suggestions that scNek2 may be functionally different from NimA in fungi. The scNek2D55G mutant in our experiments was generated specifically to mimic the characterized NimA mutants. The growth of cells carrying this scNek2D55G mutant arrested at the nonpermissive temperature, similar to the homologous nimA mutant. Interestingly, the physical association between Hec1 and scNek2D55G or Nek2E38G remained intact at any temperature, although the kinase activity of the mutants, and therefore their ability to phosphorylate Hec1, was temperature-sensitive. Hec1 appears to be a crucial substrate of Nek2, and the phosphorylation of Hec1 by Nek2 is required for passage through mitosis and for faithful chromosome segregation. These results support the notion that scNek2/kin3 is an important gene in yeast with functions similar to Nek2.

Surprisingly, a complete lack of Nek2 was not lethal in yeast; another kinase was apparently able to supplant the function of Nek2 in phosphorylating Hec1. To reconcile the apparent paradox of these observations (i.e. that precisely phosphorylated Hec1 is essential for yeast mitosis but that the kinase responsible for the phosphorylation is not), the existence other kinases with functions redundant for Nek2 must be postulated. Cdc5, based on its structural similarity with Nek2 and cell cycle expression pattern (34–36), is a potential candidate. A search of GenBank™ showed that Cdc5 shares 35% similarity with NIMA, Nek2, and scNek2/kin3 in the catalytic domain and, like Nek2, contains a coiled-coil domain near the catalytic domain. Our preliminary results have shown that Cdc5 phosphorylates Hec1 in vitro and specifically associates with Hec1 only when Nek2 is unavailable (data not shown). However, Cdc5 seems to have lower affinity for binding to Hec1. It is particularly interesting that the temperature-sensitive scNek2/kin3 mutants bind to Hec1 but fail to phosphorylate it at the nonpermissive temperature. These preliminary results suggest that binding and kinase activity are two distinct and potentially independent steps in the activation of Hec1 by Nek2. scNek2D55G thus serves as a dominant negative mutant that binds to Hec1 at the nonpermissive temperature. The secondary kinase, perhaps Cdc5, may fail to compete successfully for binding in the presence of wild-type or mutant Nek2. Cells carrying the dominant negative Nek2 mutation are markedly prone to segregation errors, however, particularly chromosomal losses (1:0 errors), perhaps because the redundant kinase for Hec1 is less efficient or less precisely regulated than Nek2. These data provide a reasonable explanation for why yeast cells completely lacking scNek2 are viable but those with the scNek2D55G mutation are growth-arrested at the nonpermissive temperature.

Implications in Higher Organisms and in Cancer—We have demonstrated that Hec1 is an important substrate of hsNek2 and scNek2/kin3. Hec1 is specifically phosphorylated by these kinases, and such phosphorylation is required for faithful chromosome segregation. Without Hec1, chromosomes distribute to daughter cells in a disordered, ultimately lethal fashion. Without precise regulation through phosphorylation of Hec1 by Nek2 during G2/M phases, more subtle errors in chromosome segregation, similar to those involved commonly in the progression of cancer in humans, are likely. If we can extrapolate findings in yeast to similar systems controlling mitosis in humans, then phosphorylation of Hec1 by Nek2 in mammalian systems may be a focus for exploring chromosomal mechanisms of carcinogenesis and cancer progression. Because of the abundant expression of Hec1 in cancer cells (1), the specific phosphorylation of Hec1 by Nek2 may also be a potential target for drug development in the treatment of cancers.

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REFERENCES
1. Chen, Y., Riley, D. J., Chen, P.-L., and Lee, W.-H. (1997) Mol. Cell. Biol. 17, 6048–6056
2. Zheng, L., Chen, Y., Riley, D. J., Chen, P.-L., and Lee, W.-H. (2000) Mol. Cell. Biol. 20, 3529–3537
3. Chen, Y., Riley, D. J., Chen, P.-L., and Lee, W.-H. (1997) Mol. Cell. Biol. 17, 6048–6056

| Yeast strain | Genotype | Total colony number | Chromosome segregation events | Significance versus weight | Significance versus weight |
|-------------|-----------|---------------------|-------------------------------|---------------------------|---------------------------|
| WHL2003*   | scHEC1/scHEC1 scNek2/scNek2 | 10,021 (0.02%) | X² = 0.00203 (NS) | 1 (0.01%) | |
| WHL4003    | Δsche1/Δsche1 hsHEC1/hsHEC1 scNek2/scNek2 | 10,483 (0.02%) | X² = 0.0035 (NS) | 1 (0.02%) | X² = 0.290 (NS) |
| WHL4001    | Δschecl/Δschecl hsHEC1S165E/ hsHEC1S165E scNek2/scNek2 | 5,748 (0.14%) | X² = 8.54 (p = 0.0035) | 1 (0.02%) | X² = 0.159 (NS) |
| WHL6502    | ΔscHEC1/scHEC1 ΔscNek2/ΔscNek2 | 6,770 (7.42%) | X² = 756 (p < 0.0001) | 4 (0.06%) | X² = 1.83 (NS) |

* Adapted from Ref. 3.
Chromosome Segregation Requires Nek2 to Phosphorylate Hec1

3. Zheng, L., Chen, Y., and Lee, W.-H. (1999) Mol. Cell. Biol. 19, 5417–5428
4. Hyland, K. M., Kingsbury, J., Koshland, D., and Hieter, P. (1999) J. Cell Biol. 145, 15–28
5. Chen, Y., Sharp, Z., and Lee, W.-H. (1997) J. Biol. Chem. 272, 24081–24087
6. Wigge, P. A., Jensen, O. N., Holmes, S., Snies, S., Mann, M., and Kilmartin, J. V. (1998) J. Cell Biol. 141, 967–977
7. Osmani, S. A., Pu, R. T., and Morris, N. R. (1988) Cell 53, 237–244
8. Osmani, S. A., Engle, D. B., Donnan, J. H., and Morris, N. R. (1988) Cell 52, 241–251
9. Osmani, S. A., May, G. S., and Morris, N. R. (1987) J. Cell Biol. 104, 1495–1504
10. Oakley, B. R., and Morris, N. R. (1983) J. Cell Biol. 96, 1155–1158
11. Chen, A., Yanai, A., Arama, E., Kilfin, G., and Motro, B. (1999) Gene (Amst.) 234, 127–137
12. Holland, P. M., Milne, A., Garka, K., Johnson, R. S., Willis, C., Sims, J. E., Rauch, C. T., Bird, T. A., and Virca, G. D. (2002) J. Biol. Chem. 277, 16229–16240
13. Letwin, K., Mizzon, L., Metz, B., Ben-David, Y., Bernstein, A., and Pawson, T. (1992) EMBO J. 11, 3521–3531
14. Li, M. Z., Yu, L., Liu, Q., Chu, J. Y., and Zhao, S. Y. (1999) Cytogenet. Cell Genet. 87, 271–272
15. Lu, K. P., and Hunter, T. (1998) Cell 91, 413–424
16. Schultz, S. J., Fry, A. M., Sutterlin, C., Ried, T., and Nigg, E. A. (1994) Cell Growth Differ. 5, 625–635
17. Tanaka, K., and Nigg, E. A. (1999) J. Biol. Chem. 274, 13491–13497
18. Schultz, S. J., and Nigg, E. A. (1993) Cell Growth Differ. 4, 821–830
19. Fry, A. M., Schultz, S. J., Bartek, J., and Nigg, E. A. (1996) J. Biol. Chem. 270, 12899–12905
20. Fry, A. M., Meraldi, P., and Nigg, E. A. (1998) EMBO J. 17, 470–481
21. Kim, Y. H. (2001) J. Arthroplasty 16, 730–739
22. Barton, A. B., Davies, C. J., Hutchinson, C. A. D., and Kahl, E. A. (1992) Gene (Amst.) 117, 157–140
23. Jones, D. G., and Rosamond, J. (1990) Gene (Amst.) 90, 87–92
24. Schweitzer, B., and Philipp, P. (1992) J. Mol. Biol. 234, 164–167
25. Chen, Y., Farmer, A. A., Chen, C.-F., Jones, D. C., Chen, P.-L., and Lee, W.-H. (1996) Cancer Res. 56, 5168–5172
26. Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. Y., and Lee, W.-H. (1989) Cell 58, 1193–1198
27. Koshland, D., and Hieter, P. (1987) Methods Enzymol. 155, 351–372
28. Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113–130
29. Smith, G. E., Summers, M. D., and Fraser, M. J. (1983) Mol. Cell. Biol. 3, 2156–2165
30. Boyle, W. J., Van Der Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–115
31. Songyang, Z., Lu, K. P., Kwon, Y. T., Tsai, L. H., Filho, O., Cochet, C., Brickley, D. A., Soderling, T. R., Bartleson, C., Graves, D. J., DeMaggio, A. J., Hoekstra, M. F., Blenis, J., Hunter, T., and Cantley, L. C. (1996) Mol. Cell. Biol. 16, 6486–6493
32. Shirayama, M., Zachariae, W., Cook, B., and Nasmyth, K. (1998) EMBO J. 17, 4336–4349
33. Charles, J. F., Jaspersen, S. L., Tinker-Kulberg, R. L., Hwang, L., Szidon, A., and Morgan, D. O. (1998) Curr. Biol. 8, 497–507
34. Cheng, L., Hunke, L., and Hardy, C. F. J. (1998) Mol. Cell. Biol. 18, 7369–7370
35. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492