Nak1, an Essential Germlinal Center (GC) Kinase Regulates Cell Morphology and Growth in Schizosaccharomyces pombe*

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Timothy Y. Huang‡, Nancy A. Markley§, and Dallan Young¶
From the Department of Biochemistry & Molecular Biology, University of Calgary, Calgary, Alberta T2N 4N1, Canada

We have identified and characterized Nak1, a 652-amino acid NH₂-terminal kinase belonging to the group II germlinal center kinase (GCK) family, in Schizosaccharomyces pombe. We found that nak1 is essential for cell proliferation. Furthermore, partial repression of nak1, under regulation of an integrated nmt1 promoter, resulted in an aberrant round cellular morphology, actin and microtubule mislocalization, slow growth, and cell division defects. Overexpression of either a kinase-inactive mutant (Nak1K93R) or the non-catalytic domain resulted in similar phenotypes, suggesting dominant-negative effects. By deletion analysis, we mapped the region responsible for this dominant-negative effect to the COOH-terminal 99 residues. Furthermore, we found that deletion of the COOH-terminal 99 residues inhibited Nak1 autophosphorylation, and expression of a partially inactive (Nak1T171A) or truncated (Nak11–562) protein only weakly suppressed morphological and growth phenotypes, indicating that both kinase and COOH-terminal regions are important for Nak1 function. GFP-Nak1 localized uniformly throughout the cytoplasm, unlike many other proteins which influence cell polarity that preferentially localize to cell ends. Together, our results implicate Nak1 in the regulation of cell polarity, growth, and division and suggest that the COOH-terminal end plays an important role in the regulation of this kinase.

Schizosaccharomyces pombe are cylindrical shaped cells that elongate by polarized growth at the cell ends during interphase. The establishment of polarity and symmetrical directionality is essential in growth and developmental processes of most eukaryotic cell types (1–4). The study of various model systems has identified many genetic elements involved in providing cells with positional information during growth and development. For example, studies of cell morphogenic processes in fission yeast have led to the identification of numerous proteins such as Cdc42 (5), Scd1 (6), casein kinase II (7), Tea1 (8), Orb6 (9, 10), and Pom1 (11) that are involved in mediating polar cell growth. Through such studies it is evident that polarized cell growth involves the coordinated function of positional signals within the cell, regulation of signal transduction pathways, and cytoskeletal reorganization (3, 12). However, the mechanisms by which such proteins regulate cell morphogenesis and the mode by which their dysfunction results in loss of cell polarity remain unclear.

The p21-activated kinases (PAKs) have been implicated in the regulation of cell morphology and cytoskeletal dynamics (13), various signaling pathways (14–19), and apoptotic responses (20, 21). PAK-related kinases are grouped into two main families based on the arrangement of their respective functional domains (22, 23). The true PAKs, originally characterized as primary downstream effectors for Rac/Cdc42 small molecular weight GTPases, have a COOH-terminal kinase domain and an NH₂-terminal regulatory region. The NH₂-terminal domain contains a conserved CRIB (Cdc42/Rac interactive binding) motif that mediates Cdc42/Rac binding to PAKs, resulting in their consequent activation (24, 25). In fission yeast, Shk1/Pak1 is a critical effector for Cdc42, and it has been shown to play roles in the regulation of cell morphology, sexual differentiation, and mitosis (16, 26–29). Genetic analyses suggest that the functions of the two fission yeast PAKs (Pak1/Shk1 and Pak2/Shk2) are largely redundant (29, 30).

A second PAK-related kinase family, the GCKs (germlinal center kinases), comprise highly conserved NH₂-terminal kinase domains and less conserved COOH-terminal regulatory domains. Unlike the true PAKs, GCKs do not have CRIB motifs and do not bind Rac/Cdc42 GTPases. GCKs can be subdivided into two groups based on their structural and functional properties. Group I GCKs are most similar to mammalian Gck1 and have homologous carboxyl termini containing at least two PEST motifs, two polyproline SH3 domain binding sites, and an additional ~350-amino acid highly conserved region (22). Various group I GCKs have been implicated in mediating stress response and cytoskeletal arrangement (22, 23, 31). The function and regulation of group II GCKs are less well characterized. Studies in fission yeast have shown that Sid1, a group II GCK, is essential in mediating cytokinesis, most likely by localizing and phosphorylating downstream targets at spindle pole bodies during anaphase and septation (32, 33). Cdc14 was recently shown to positively regulate Sid1 by binding the COOH-terminal region of this kinase (34). Previous biochemical evidence suggested that the carboxyl-terminal region of some group II GCKs contains an autoinhibitory domain, al-

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† Supported by the National Sciences and Engineering Research Council of Canada and the Alberta Heritage Foundation for Medical Research.

§ Supported by the Alberta Cancer Board.

¶ Supported by the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed: Dept. of Biochemistry & Molecular Biology, University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta T2N 4N1, Canada. Tel.: 403-220-3030; Fax: 403-283-8727; E-mail: young@ucalgary.ca.

The abbreviations used are: PAK, p21-activated kinase; CRIB, Cdc42/Rac interactive binding; GCK, germlinal center kinase; HA, hemagglutinin; DIC, differential interference contrast; CTR, COOH-terminal region; GFP, green fluorescent protein; SH, Src homology domain; TRITC, tetramethylrhodamine isothiocyanate.
though Sid1 requires the regulatory region for optimal activity (34–36).

In this paper, we describe the identification and characterization of Nak1, a fission yeast group II GCK. Our results indicate that Nak1 is required for bipolar cell morphology and cell growth and suggest that the COOH-terminal region plays an important role in the regulation of this kinase.

EXPERIMENTAL PROCEDURES

Yeast Strains and Methods—Genotypes of *S. pombe* strains used in this study are listed in Table I. Methodology employed in cultivating yeast strains, transformation, iodine staining, and transformations was undertaken according to procedures described previously (37).

Identification of nak1—A DNA fragment encoding an NH2-terminal Nak1 peptide was amplified from a *S. pombe* cDNA library, as described previously (38). The amplified *nak1* DNA fragment was used as a probe to isolate several clones containing the *nak1* gene from a *S. pombe* genomic DNA library constructed in the pWH5 vector (gift of D. Beach); a 7-kb *Xba*I DNA fragment encoding the entire *nak1* gene was subcloned into Bluescript II SK− (Stratagene) to generate pNak1. The *nak1* DNA sequence was determined and has been deposited in the GenBankTM database (accession number AF091345).

Plasmids—Techniques used in PCR amplification, restriction digestion, and other cloning procedures have been described previously (39). pREP3XHA-Nak1, pREP3XHA-Nak1K39R, and pREP3XHA-Nak1481 were constructed by amplifying the *nmt1*-coding fragments from *pREP3XHA*-Nak1, *pREP3XHA*-Nak1K39R, and *pREP3XHA*-Nak1481 by PCR and ligated into the SpeI/BamHI sites of *pREP3XHA*-H1 sites of pREP3XHA (40). pREP3XHA-Nak1K39R was constructed by (i) amplification of Nak1 using the mutagenic primer 5′-AAATTAAGATCCGATGGCAACAG and the *T1* forward primer 5′-GCGGCTCAGTATGGAAAAATACACTGTCCT; (ii) the 128-bp product was used to amplify a Nak1K39R fragment with the T5 reverse primer 5′-GGCTGAGATCTGGCTGACTATGCCAGCCTGGGAGGACCGTCGACAACT-ATGACGTGCCGACTTACGAGTTCGTTACGAGCTGGAGGACCGTCGACACCTCAGTGGCACCGGCGGATCCATGCGACATGGCACTGCGTATGACGAGGTAGGGAGGACCGGGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGAC of the appropriate mutation; and (iii) the 128-bp fragment was ligated into the SpeI/BamHI sites of *pREP3XHA*-H1 sites of *pREP3XHA*-Nak1 (40). pREP3XHA-Nak1K39R was constructed by (i) amplification of Nak1 using the mutagenic primer 5′-AAATTAAGATCCGATGGCAACAG and the *T1* forward primer 5′-GCGGCTCAGTATGGAAAAATACACTGTCCT; (ii) the 128-bp product was used to amplify a Nak1K39R fragment with the T5 reverse primer 5′-GGCTGAGATCTGGCTGACTATGCCAGCCTGGGAGGACCGTCGACAACT-ATGACGTGCCGACTTACGAGTTCGTTACGAGCTGGAGGACCGTCGACACCTCAGTGGCACCGGCGGATCCATGCGACATGGCACTGCGTATGACGAGGTAGGGAGGACCGGGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAGGGAGGACCGGAGGAGGTACTTCAGTTACGACGAGGTAG
Nak1 Is Essential for Viability, and nak1 Repression Results in Loss of Cell Polarity and Growth Inhibition—To investigate the function of Nak1, we constructed and examined the phenotypes of S. pombe strains in which nak1 is deleted. One copy of the nak1 open reading frame was replaced with the ura4 selectable marker in a diploid S. pombe strain (see “Experimental Procedures”). Sporulation and tetrad analysis of several independently derived Ura− diploid strains consistently resulted in two viable Ura− spores, indicating that nak1 is essential for germination and/or cell growth. On closer examination, it was clear that the other two spores in each tetrad had germinated and given rise to microcolonies (2–8 cells) prior to growth arrest. The cells in these small colonies exhibited an abnormal round shape (data not shown).

To further investigate the function of Nak1, we generated a strain in which the endogenous nak1 promoter was replaced with the thiamine-repressible nmt1 promoter (see “Experimental Procedures”). We observed that 12 h after addition of thiamine to repress nak1 expression, cells were smaller and more oval-shaped, rather than exhibiting the normal rod-shaped morphology of fission yeast (Fig. 2A). This indicates that cells have begun to lose normal polarity, but suggests that they still retain sufficient levels of Nak1 to maintain partial cell polarity.

Next, we identified and determined the DNA sequence of a 7-kb DNA insert containing the entire gene (see “Experimental Procedures”). The DNA sequence of nak1 revealed three exons encoding a 652-amino acid residue protein that exhibits a high degree of sequence identity with PAKs and is most closely related to the group II GCKs (Fig. 1). Like other GCKs, Nak1 has an NH2-terminal catalytic region containing the PAK GTPY/FWMAPE signature motif and other conserved domains typical of GCKs. In addition, Nak1 contains a 411-residue COOH-terminal non-catalytic domain that does not share significant sequence identity with other proteins. Similar to group II GCKs, Nak1 lacks a CRIB CDC42/RAF-binding motif characteristic of PAKs, or SH3-binding motifs unique to PAKs and group I GCKs.

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RESULTS

Nak1 Belongs to the Group II GCK Family—We previously identified kinases from a fission yeast cDNA library by PCR using degenerate oligonucleotides derived from conserved regions of kinase catalytic domains as primers (38). The PCR products were cloned, and their DNA sequences were determined. One clone we identified encoded a region of a novel kinase that we have named Nak1. We used this clone to generate a probe to screen a S. pombe genomic DNA library, and we identified and determined the DNA sequence of a 7-kb DNA insert containing the entire nak1 gene (see “Experimental Procedures”). The DNA sequence of nak1 revealed three exons encoding a 652-amino acid residue protein that exhibits a high degree of sequence identity with PAKs and is most closely related to the group II GCKs (Fig. 1). Like other GCKs, Nak1 has an NH2-terminal catalytic region containing the PAK GTPY/FWMAPE signature motif and other conserved domains typical of GCKs. In addition, Nak1 contains a 411-residue COOH-terminal non-catalytic domain that does not share significant sequence identity with other proteins. Similar to group II GCKs, Nak1 lacks a CRIB CDC42/RAF-binding motif characteristic of PAKs, or SH3-binding motifs unique to PAKs and group I GCKs.

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Although the small cell size could reflect a reduced G2 phase, this effect appears to be temporary, since many cells become large and completely round by 18 h after addition of thiamine. Also, at the 18-h time point, actin was no longer polarized and was redistributed around the cellular circumference. We also observed that actin still localized to medial sites of division after loss of cell polarity. Also, microtubules, which normally extend along the long axis of cells, are short and appear to be scattered in differing directions within the cell 12 h after addition of thiamine. In addition, nak1-repressed cells exhibited a slow growth phenotype, which was more severe at high temperature and high salt concentration (Fig. 2B). We also examined cell cycle progression of nak1-repressed cells by flow cytometry. Actively growing fission yeast spend ~70% of their time in G2, with a 2C DNA content. Nuclear division occurs in M phase resulting in binucleated cells, which enter the G1 phase of the cell cycle. However, cell division is not normally completed until S phase, as DNA replication takes place. Thus, most cells contain 2C DNA content, except during S phase when the DNA content varies between 1C (cells that divide before DNA replication) and 4C (cells that remain undivided after DNA replication) (48, 49). Interestingly, we found that a large population of cells with greater than 2C DNA content accumulated upon nak1 repression (Fig. 2C), suggesting a delay in cell division or cell separation until late S phase. This conclusion is further supported by our observation that there is a concomitant increase in the proportion of binucleated cells upon nak1 repression (Table II). Previous reports indicate that ~10% of wild-type cells are septated/binucleated in a growing culture (50, 51). We observed a similar level of nmt1-nak1 binucleated cells in the absence of thiamine, but this level increased significantly 12–18 h after the addition of thiamine to repress nak1 expression. A significant proportion of these binucleated cells were septated/binucleated in a growing culture (50, 51). We observed that Nak1 localizes uniformly in the cytoplasm, cell proliferation, and normal cell cycle progression.

Overexpression of a Mutant Kinase-inactive Nak1 or the Non-catalytic Domain Results in Dominant-negative Phenotypes—To further examine the function and regulation of Nak1, we generated expression constructs containing a series of deletions and specific mutations within the Nak1 coding sequence (Fig. 3), including mutation of the critical ATP-binding region (HA-Nak1K39R) predicted to produce an inactive kinase. We also generated a construct predicted to encode a partially inactive kinase (HA-Nak1T171A) by site-directed mutation of a critical autophosphorylation/activation Thr residue (19). These mutant proteins were detectable in yeast extracts by Western blot analysis using anti-HA antibody, indicating that they were stably expressed (data not shown). We found that overexpression of HA-Nak1 in a normal haploid S. pombe background did not have a significant effect on growth or morphology (data not shown). However, overexpression of either the kinase-inactive mutant (HA-Nak1K39R) or the non-catalytic domain of Nak1 (HA-Nak1T171A) produced a round morphology similar to that resulting from nak1 repression (Fig. 4A), suggesting that these mutant proteins act in a dominant-negative manner to block endogenous Nak1 function. Furthermore, overexpression of the partially inactive mutant HA-Nak1T171A produced a similar dominant-negative morphological effect (data not shown). These morphological aberrations are also associated with slow growth at high temperature and high salt concentration (35 °C, 1.2 M KCl) (Fig. 4B).

The causative region of Nak1 producing these dominant-negative morphological and proliferative phenotypes was mapped to the COOH-terminal end (residues 554–652), which we refer to as the CTR (COOH-terminal region). Expression of the CTR (HA-Nak1554–652) alone was sufficient to produce a dominant-negative morphological phenotype, while cells expressing the Nak1 non-catalytic domain lacking the CTR (HA-Nak1562–652) appeared normal (Fig. 4A). Moreover, expression of kinase-inactive Nak1 (HA-Nak1K39R), the non-catalytic domain (HA-Nak1554–652), or the CTR (HA-Nak11481–652) resulted in severe growth inhibition at high temperature and high salt concentration (35 °C, 1.2 M KCl), but cells expressing the kinase-inactive mutant lacking the CTR (HA-Nak11554–652, K39R) or the non-catalytic domain lacking the CTR (HA-Nak11562–652) were able to grow under these conditions (Fig. 4B). In summary, these results suggest that overexpression of the CTR abrogates endogenous Nak1 activity resulting in dominant-negative phenotypes.

The CTR Is Required for Proper Nak1 Function—Expression of HA-Nak1 results in full reversion of morphological and slow growth phenotypes associated with nak1 repression in nmt1-nak1 strains (Fig. 5, A and B). However, expression of the partially inactive mutant (HA-Nak1T171A) or Nak1 lacking the CTR (HA-Nak1562–652) only partially rescued the morphological and slow growth phenotypes. These results suggest that both kinase activity and the CTR are important for Nak1 function.

To further examine the role of the CTR, we expressed HA-Nak1 and HA-Nak11562–652 in a wild-type S. pombe strain, immunoprecipitated the HA-tagged proteins from cell extracts, and performed in vitro kinase assays. We found that immunoprecipitated HA-Nak1 exhibited a detectable level of autophosphorylation and phosphorylation of myelin basic protein, whereas deletion of the CTR resulted in a significant decrease in kinase activity, indicating that the CTR is critical for Nak1 kinase activity in vitro (Fig. 5C). Together, our evidence implicates the CTR as an important regulatory sequence and suggests that Nak1 may be regulated by the association of key factors with the CTR.

Nak1 Localizes Uniformly in the Cytoplasm—To examine the localization of Nak1 we constructed vectors to express GFP-
tagged Nak1 from the adh1 promoter. Expression of GFP-Nak1 rescued growth and morphological defects in nak1-repressed strains, indicating that it is functional (data not shown). Visualization of various GFP-Nak1 mutants expressed in wild-type cells revealed that GFP-Nak1, GFP-Nak11–562 (lacking the CTR), GFP-Nak1481–652 (the CTR alone), and the GFP control localized throughout the cytoplasm (Fig. 6). Although other kinases, which regulate cell polarity in fission yeast, localize to cell ends in the presence of high salt (52), GFP-Nak1 localization was observed to be unaffected in the presence of 1.2 M KCl (data not shown). Interestingly, we found that GFP-Nak1 and GFP-Nak11–562 were excluded from the nucleus, whereas the GFP control and GFP-Nak1 481–652 constructs localized throughout the cell including the nucleus. However, GFP-Nak1481–652 constructs were consistently more concentrated around the nucleus. Although the significance of this observation is unclear, it may reflect active cellular mechanisms that exclude Nak1 from the nucleus and localize the Nak1 CTR to regions around the nucleus.

DISCUSSION

Nak1 Is a GC Kinase That Mediates Cell Growth and Morphology in S. pombe—In this study, we report the identification and characterization of the Nak1, a protein kinase in S. pombe. The lack of proline-rich SH3-binding motifs within the COOH-terminal non-catalytic domain of Nak1 indicates that Nak1 belongs to the group II subclass of GCKs. In addition to Nak1,
that a region (the CTR) within the non-kinase domain of Nak1 is essential for Nak1 function as measured by in vitro kinase assays and functional complementation of nak1-repressed growth and morphological defects. Furthermore, overexpression of the CTR produced dominant-negative morphological and growth inhibitory phenotypes. This dominant-negative effect may result from the ineffectual binding of positive regulators of Nak1 to the CTR.

Together, our results indicate that Nak1 is essential for cell growth, polarity, and normal cell division, and the CTR is important for Nak1 function. Further studies of the interactions between Nak1 and associated proteins may provide insight into the regulation of Nak1-dependent morphogenetic mechanisms.

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Timothy Y. Huang, Nancy A. Markley and Dallan Young

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