Keywords: fission yeast, casein kinase 2, Ste20 kinase, polar growth

Submitted: 02/22/13
Accepted: 02/24/13
http://dx.doi.org/10.4161/cc.24095

*Correspondence to: Dannel McCollum and Juraj Gregan; Email: Dannel.McCollum@umassmed.edu and juraj.gregan@univie.ac.at

Although the sterile 20 (Ste20) serine/threonine protein kinase was originally identified as a component of the *S. cerevisiae* mating pathway, it has homologs in higher eukaryotes and is part of a larger family of Ste20-like kinases. Ste20-like kinases are involved in multiple cellular processes, such as cell growth, morphogenesis, apoptosis and immune response. Carrying out such a diverse array of biological functions requires numerous regulatory inputs and outputs in the form of protein-protein interactions and post-translational modifications. Hence, a thorough knowledge of Ste20-like kinase binding partners and phosphorylation sites will be essential for understanding the various roles of these kinases. Our recent study revealed that *Schizosaccharomyces pombe* Nak1 (a conserved member of the GC-kinase sub-family of Ste20-like kinases) is in a complex with the leucine-rich repeat-containing protein Sog2. Here, we show a novel and unexpected interaction between the Nak1-Sog2 kinase complex and Casein kinase 2 (Cka1, Ckb1 and Ckb2) using tandem-affinity purification followed by mass spectrometric analysis. In addition, we identify unique phosphosites on Nak1, Sog2 and the catalytic subunit of casein kinase 2, Cka1. Given the conserved nature of these kinases, we expect this work will shed light on the functions of these proteins both in yeast and higher eukaryotes.
Extra View

Results and Discussion

In order to identify proteins that physically interact with the Nak1 kinase, we constructed functional tandem affinity purification (TAP)-tagged Nak1 (Nak1-TAP) according to our protocol described at http://mendel.imp.ac.at/Pombe_tagging/. We used a TAP protocol to purify Nak1-TAP, and co-purifying proteins were identified by mass spectrometry. Among proteins with at least two unique peptides, we identified leucine-rich repeat protein Sog2, an uncharacterized protein SPBC2G5.02c, as well as catalytic (Cka1) and regulatory subunits (Ckb1) of casein kinase 2 (Fig. 1A). To confirm the specificity of these interactions, we also performed reciprocal purifications using functional TAP-tagged Sog2 (Sog2-TAP) and Cka1 (Cka1-TAP). Indeed, we found that Nak1, Cka1, Ckb1 and SPBC2G5.02c co-purify with Sog2-TAP and Nak1, Sog2, Ckb1 and SPBC2G5.02c co-purify with Cka1-TAP (Fig. 1A). Our finding that Nak1 co-purifies with Sog2 is consistent with our recent study15 and the observation from budding yeast that the Sog2 ortholog is known to interact with the Nak1 ortholog Kic1.17 More intriguing is our observation that Nak1 co-purifies with casein kinase 2 (CK2) (Fig. 1A). CK2 is an evolutionarily conserved serine/threonine protein kinase that regulates many cellular processes, including cell signaling and proliferation, DNA repair, apoptosis and senescence.18-22 Extensive evidence of its involvement in various cancers as well as neurodegenerative diseases had led to its emergence as a promising

Figure 1. Identification and characterization of proteins co-purifying with Nak1-TAP, Sog2-TAP and Cka1-TAP. (A) List of proteins identified by mass spectrometry co-purifying with *S. pombe* Nak1-TAP, Sog2-TAP and Cka1-TAP. Proteins were purified from cycling *S. pombe* cells expressing Nak1-TAP (JG15615), Sog2-TAP (JG16552) or Cka1-TAP (JG15429). Only proteins identified with at least two peptides are included. For a full list of identified proteins see Table S1. Proteins found in other unrelated purifications (common contaminants) are omitted from this table. Number of unique peptides and sequence coverage are indicated. (B) Summary of phosphorylation sites identified by mass spectrometry. Protein purifications were performed as described in (A). Phosphorylation sites identified on Nak1, Cka1 and Sog2 in this study are indicated. New phosphorylation sites identified are indicated in bold. Sites with the R-X-X-S consensus motif are indicated in red. Asterisks indicate sites with the CK2 consensus motif S/T-X-X-D/E.

regulation of cell growth and division.10,14 Specifically, there appears to be mutual antagonism between the SIN and MOR pathways, whereby the SIN inhibits MOR signaling through phosphorylation of the Nak1 kinase.10,14 Nak1 is in a complex with the leucine-rich repeat-containing protein, Sog2, which might also be a target of SIN inhibition.15 Therefore, studying Nak1 phosphorylation and interacting proteins has provided insights into cross-talk between conserved NDR-kinase signaling networks.

Interestingly, the genetic screen for cell polarity mutants, which identified Nak1 and other MOR pathway components, also identified mutants in casein kinase 2.30 However, how casein kinase 2 fits in to the MOR signaling pathway has remained mysterious.
are indicative of a loss of bipolar growth.30 However, crosstalk between protein kinases from the Ste20 family and CK2 kinases is poorly understood.

So far, the only evidence linking these two kinase families in humans appears to be from Chaa et al., who observed that human CK2 can phosphorylate the Ste20-like kinase family member SLK in vitro.24 It has also been shown that CK2 subunits in S. cerevisiae interact with either Ste20 and/or with Bem1, a cell polarity establishment protein that acts as a scaffold for Ste20.25,26 Few other studies in yeast and humans show interaction of CK2 subunits with Ste20-associated proteins. For instance, budding yeast Cka1 subunit associates with Ste20-associated proteins. For instance, budding yeast Cka1 interacts with Tea1, a microtubule-associated polarity protein that is regulated through phosphorylation by the Pak1/Shk1 kinase.29 Together, it suggests that CK2 affects Ste20-mediated signaling to regulate processes such as polarized growth. Moreover, mutations in both nak1 and cka1 genes in S. pombe result in a similar phenotype—spherical cells, which are indicative of a loss of bipolar growth.30

We also observed that Cka1-GFP localizes to the nucleolus, cell tips, and the spindle pole bodies (Fig. 2A and B). While Nak1 localizes to the SPB only during early mitosis, Cka1-GFP appears to remain at the SPBs at all times. Therefore, it partially co-localizes with Nak1 at the SPB. Similar to Nak1, Cka1 also localizes to the cell tips and the septum (Fig. 2A). This reinforces the possibility that crosstalk between Nak1 and Cka1 kinases is important for the establishment of polar growth, and it will be interesting to investigate this in the future.

Protein kinase CK2 is a tetramer composed of two catalytically active (CK2 α isoforms) and two regulatory (CK2 β isoforms) subunits. In fission yeast, one catalytic subunit (Cka1) and one regulatory subunit (Ckb1) have been characterized.31 Our analysis showed that Cka1, in addition to Ckb1, co-purifies with an uncharacterized protein SPBC2G5.02c (Fig. 1A). Our sequence searches revealed that SPBC2G5.02c shows high sequence similarity to Ckb1 as well as to other CK2 β isoforms from various species (data not shown). This suggests that SPBC2G5.02c gene encodes for the second fission yeast CK2 β isoform, and we propose to call this gene ckb2 (CK2 beta isoform 2).

Next, we mapped the phosphorylation sites on proteins identified in our purifications. Nak1 was phosphorylated on 22 residues, Sog2 on 19 residues and Cka1 on a single residue (Fig. 1B). Previous studies found 15 residues phosphorylated on Nak1 (S373, S376, S383, S386, S407, T446, S448, S450, S479, S481, S539, S541, S543, S549, S552) and 11 residues phosphorylated on Sog2 (S301, T392, T395, S398, S400, T403, S404, S464, S648, S829, S839). In this study, we show 11 and nine new phosphosites on Nak1 and Sog2, respectively (Fig. 1B, indicated in bold). Sites with an RXXS consensus motif may be targets of the Cdk1 kinase.34 Furthermore, both Nak1 and Sog2 contain the consensus phosphorylation motif S/T-X-X-D/E, which is recognized by CK2.23,35 Some of these sites have been identified by our analysis (Fig. 1B, indicated by asterisks). This indicates a possibility that Nak1 and Sog2 may be potential CK2 substrates. However, further mutational studies will be required to confirm this. Because most of the phosphorylation sites on Nak1 cluster in the C-terminal non-kinase half of the protein, we expect that this region is a key regulatory module targeted by multiple kinases.

Taken together, our results presented in this study provide a thorough biochemical

**Figure 2.** Cka1-GFP localizes similar to MOR pathway proteins like Nak1. (A) Fixed Cka1-GFP-expressing cells (YDM2969) were stained with DAPI. White arrows in the merge panel indicate localization of Cka1-GFP to dots that might correspond to the SPB. Red arrows indicate Cka1-GFP localization to cell septum and tips. (B) Cka1 co-localizes with the SPB marker Sad1. Fixed Cka1-GFP Sad1-RFP expressing cells (YDM3463) were stained with DAPI.
analysis of Nak1-interacting partners and their post-translational modifications. We confirmed that Nak1 co-purifies with Sog2. Significantly, Nak1 shows interaction with the CK2 complex. We therefore established a physical interaction between members of two major kinase families, namely Ste20-related kinases and the Casein kinase 2 family. Further exploration should reveal a better understanding of the functional relationship between them.

Materials and Methods

Strains. S. pombe strains expressing either Nak1-TAP (JG15615, b' nak1-TAP::KanMX), Cka1-TAP (JG15429, b' cka1-TAP::KanMX) or Sog2-TAP (JG16552, b' sog2-TAP::KanMX) were grown in complete yeast extract medium (YE + 5S). TAP-tagging was confirmed by PCR and immunoblotting. The TAP epitope was detected using PAP linked to peroxidase, Dako) at 1:50,000. Proteins were extracted using IPP150 buffer and with 5 volumes of 1 M CaCl₂ and mixed with 6 ml of Calmodulin Sepharose™ 4B beads (GE Healthcare, cat. # 17-0529-01) was washed with CBB1 buffer and incubated for 2 h at 4°C. The beads were washed with 10 volumes of CBB1 and 5 volumes of CBB2, 10 mM Tris pH 8.0, 150 mM NaCl, 1 mM Mg-Acetate, 2 mM CaCl₂, 1 mM β-mercaptoethanol. The proteins were step-eluted using bead volume of elution buffer (EB, 10 mM Tris pH 8.0, 150 mM NaCl, 1 mM Mg-acetate, 2 mM EGTA, 1 mM β-mercaptoethanol). Eluted proteins were separated on SDS-PAGE and checked by silver staining. The pH of the elution buffer was adjusted to 8.5. Disulfide bonds were reduced with DTT (1:2 of the estimated amount of protein) for 30 min at 56°C and subsequently alkylated with iodoacetamide (1:2 of the estimated amount of protein) for 20 min at RT protected from light. DTT (1:20 of the estimated amount of protein) was added to consume excess iodoacetamide and proteins were digested either with trypsin (recombinant, proteomics grade, Roche; 1:25 of the estimated amount of protein) at 37°C overnight. Digestion was stopped by addition of trifluoroacetic acid to pH 2.

Digests were separated on an UltiMate 3000 RSLC nano LC system (Dionex, Thermo Fisher Scientific). Peptides were loaded on a trapping column (PepMap C18, 5 μm particle size, 300 μm i.d. × 5 mm, Dionex, Thermo Fisher Scientific) equilibrated with 0.1% TFA and separated on an analytical column Acclaim PepMap RSLC C18 (50 cm × 75 μm × 2 μm, 100 Å, Dionex, Thermo Fisher Scientific) applying a 30 min resp. 60 min linear gradient from 2% up to 40% acetonitrile. The HPLC was directly coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) via a nano-electrospray ionization source (Proxeon, Thermo Fisher Scientific). The electrospray voltage was set to 1,900 V. The mass spectrometer was operated in the data-dependent mode: one full scan (m/z: 350–2,000, resolution 70,000) with lock mass enabled was followed by maximal 10 MS/MS scans. The lock mass was set at the signal of poly(dimethyl)cyclohexane at m/z 445.12005. The 10 most intense ions were fragmented by higher energy collisional dissociation (HCD) with normalized collision energy of 30. Fragment spectra were acquired with a resolution of 17,500. The ion target value for full MS was set to 1,000,000 for MS/MS to 100,000. Fragmented ions were excluded from further selection for 20 sec.

Enzymatic digest, LC-MS/MS analysis and data analysis. The pH of the sample was adjusted to 8.5. Disulfide bonds were reduced with DTT (1:10 of the estimated amount of protein) for 30 min at 56°C and subsequently alkylated with iodoacetamide (1:2 of the estimated amount of protein) for 20 min at RT protected from light. DTT (1:20 of the estimated amount of protein) was added to consume excess iodoacetamide and proteins were digested either with trypsin (recombinant, proteomics grade, Roche; 1:25 of the estimated amount of protein) at 37°C overnight. Digestion was stopped by addition of trifluoroacetic acid to pH 2.

Acknowledgments

This work was supported by Austrian Science Fund grants P23609, P21437 and P3403, HFSP grant RGY0069/2010 and by the Slovak Research and Development Agency contract No. APVV-0111-12 and APVV-0334-12. L.C. was supported by the (European Community’s) Seventh Framework Programme (FP7/2007–2013) under grant agreement number PERG07-GA-2010-268167. J.G. was supported by the (European Community’s) Seventh Framework Programme (FP7/2007–2013) under grant agreement number PCIG11-GA-2012-322300. D.M. and S.G. were supported by the National Institutes of Health grant GM058406-14 to D. McCollum.