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Lubos Cipak
University of Vienna

Sneha Gupta
University of Massachusetts Medical School, sneha.gupta@umassmed.edu

Iva Rajovic
University of Vienna

See next page for additional authors

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Crosstalk between casein kinase II and Ste20-related kinase Nak1

Lubos Cipak,1,2,† Sneha Gupta,3,† Iva Rajovic,1 Quan-Wen Jin,3,4 Dorothea Anrather,1 Gustav Ammerer,1 Dannel McCollum3,* and Juraj Gregan1,5,*

1Max F. Perutz Laboratories; Department of Chromosome Biology; University of Vienna; Vienna, Austria; 2Cancer Research Institute; Slovak Academy of Sciences; Bratislava, Slovak Republic; 1Department of Microbiology and Physiological Systems and Program in Cell Dynamics; University of Massachusetts Medical School; Worcester, MA USA; 4Department of Biological Medicine; School of Life Sciences; Xiamen University; Xiamen, China; 5Department of Genetics; Comenius University; Bratislava, Slovak Republic

†These authors contributed equally to this work.

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.
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. Nak1 is in a complex with the leucine-rich repeat-containing protein, Sog2, which might also be a target of SIN inhibition. 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. How casein kinase 2 fits into the MOR signaling pathway has remained mysterious.

## 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 study and the observation from budding yeast that the Sog2 ortholog is known to interact with the Nak1 ortholog Kic1. 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. Extensive evidence of its involvement in various cancers as well as neurodegenerative diseases had led to its emergence as a promising
drug target. 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 Chaar et al., who observed that human CK2 can phosphorylate the Ste20-like kinase family member SLK in vitro. 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. 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 target Stel1 (MAP3K). The Csk2B subunit of human CK2 contains the binding domain for p21 (CDKN1A), which is required for p21-mediated CK2 inhibition. In *S. pombe*, CK2 subunits Cka1 and Ckb1 interact with Tea1, a microtubule-associated polarity protein that is regulated through phosphorylation by the Pak1/Shk1 kinase. 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.

We also observed that Cka1-GFP localizes to the nucleolus, cell tips, septa 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 (CK2α isofoms) and two regulatory (CK2β isofoms) subunits. In fission yeast, one catalytic subunit (Cka1) and one regulatory subunit (Ckb1) have been characterized. 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 CK2β isoforms from various species (data not shown). This suggests that SPBC2G5.02c gene encodes for the second fission yeast CK2β 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 are targeted by NDR kinases. We previously showed that the RXXS sites on Nak1 are phosphorylated by the SIN pathway NDR kinase, Sid2. All Sid2 phosphorylation sites identified by this study were mutated previously (ref. 15 and unpublished data; Fig. 1B, shown in red). Analysis of the mutants showed that Sid2 phosphorylation of Nak1 causes removal of Nak1 from the spindle pole bodies, which prevents premature activation of the SIN in early mitosis. Moreover, Sid2 phosphorylation of Nak1 blocks MOR signaling by preventing interaction of Nak1 with the scaffold protein Mor2. The presence of RXXS site phosphorylation on the Nak1 binding partner Sog2 suggests that it might also be phosphorylated by the SIN kinase Sid2. To completely understand the role of phosphorylation in the regulation of Nak1, Sog2 and Cka1, it will be important to identify relevant protein kinases and counteracting phosphatases, analyze possible periodicity during the cell cycle and study functional consequences of mutating these phosphorylation sites.

We suggest that sites with the phospho-motif pSP may be targets of the Cdk1 kinase. Furthermore, both Nak1 and Sog2 contain the consensus phosphorylation motif S/T-X-X-D/E, which is recognized by CK2. 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
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, h<sup>b</sup> nak1-TAP::KanMX), Cka1-TAP (JG15429, b<sup>r</sup> cka1-TAP::KanMX) or Sog2-TAP (JG16552, b<sup>r</sup> sog2-TAP::KanMX) were grown in complete yeast extract medium (YE + 5S) 36-38 TAP-tagging was confirmed by PCR and immunoblotting. The TAP epitope was detected using PAP antibodies (rabbit antiperoxidase antibody linked to peroxidase, Dako) at 1:50,000 dilution in PBS-T.

Protein purification. Six-liter cultures of strains expressing TAP-tagged proteins were grown to mid-log phase (OD ~0.8) and collected by centrifugation. Yeast cell powder was made from frozen pellet using SPEX SamplePrep 6870 Freezer/Mill. Proteins were extracted using IPP150 buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% NP-40, complete protease and phosphatase inhibitors, 1 mM PMSF). All washing steps were performed in Poly-Prep columns by gravity flow (Bio-Rad Laboratories, Ges.m.b.H). Five hundred ml of eluate was supplemented with 6 μl of AcTEV™ protease (Invitrogen, cat. # 12575-015) for 2 h at 16°C. Two ml of eluate was supplemented with 6 μl of M CaCl₂, and mixed with 6 ml of Calmodulin binding buffer 1 (CBB1, 10 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM imidazole, 1 mM Mg-Acetate, 2 mM CaCl₂, 10 mM β-mercaptoethanol). One hundred and fifty μl of Calmodulin Sepharose™ 4B beads (GE Healthcare, cat. # 17-0529-01) was washed with CBBI buffer, added to a mixture of eluate and CBB1 buffer and incubated for 2 h at 4°C. The beads were washed with 10 volumes of CBB1 and 5 volumes of Calmodulin binding buffer 2 (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. Eluates from peak fraction were submitted for LC-MS/MS analysis.36,39,40

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. 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 polydimethylcyclosiloxane at m/z 445.12025. 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.

Peptide identification was performed using the SEQUEST algorithm in the Proteome Discoverer 1.3.0.339 software package (Thermo Fisher Scientific). Spectra were searched against the Sanger S. pombe database (04/23/2012). Following search parameters were used: peptide tolerance was set to 10 ppm, MS/MS fragment tolerance to 50 mmu, trypsin was selected as protease and two missed cleavages were allowed, carbamidomethylcysteine was set as static modification, oxidation of methionine and phosphorylation of serine, threonine and tyrosine as a variable modification. The results were filtered at the XCorr values to an FDR of 1% on the peptide level.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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