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Characterization of the Ste20-like kinase Krs1 of *Dictyostelium discoideum*

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Abstract

Ste20-like kinases constitute a ubiquitous and expanding group of serine/threonine kinases, homologous to Ste20 in *Saccharomyces cerevisiae*. The social amoeba *Dictyostelium discoideum* contains at least 17 members of this kinase family, 13 from the germinal center kinase (GCK) subgroup and 4 p21-activated kinases (PAK). Here, we describe the kinase Krs1 which is encoded by the gene *krsA*, and phylogenetic analysis groups it into subfamily GCK-II together with human MST2 and MST1 or Hippo from *Drosophila melanogaster*. Significant similarities are found especially in the catalytic domain and in a short regulatory region (SARAH) which is thought to be important for protein/protein interactions. Northern blot analysis showed a single *krsA* transcript throughout development with an upregulation at 12 h after the onset of starvation. The protein levels as detected with anti-Krs1 polyclonal antibodies revealed a similar pattern. Gel filtration experiments suggested that AX2 wild-type cells harbored multimeric forms of Krs1. In vitro phosphorylation assays with recombinant protein showed that the kinase exhibits autophosphorylation and accepts myelin basic protein and *D. discoideum* severin as substrates. A series of C-terminal deletions of Krs1 indicated that the regulatory domain in the C-terminal half contains inhibitory elements, and highlighted the importance of two predicted α-helices following subdomain XI of the classical catalytic domain. GFP-Krs1-overexpressing wild-type cells showed an enrichment of the kinase in the cortex, and motility of these cells during aggregation was reduced. Krs1 knockout strains exhibited only subtle differences to wild-type cells which suggests a certain redundancy among Ste20-like kinases in *D. discoideum*.

Keywords: Krs1; Krs2; Kinase; *Dictyostelium*; SARAH; Chemotaxis; Phototaxis; Severin; Ste20; Knockout

Introduction

Ste20-like kinases are serine/threonine kinases found in all eukaryotic species tested so far. Their catalytic domains are highly homologous to the Ste20 kinase of *Saccharomyces cerevisiae*, the founding member of this group. The lack of Ste20 in yeast led to sterile cells due to the disruption of a MAP kinase pathway downstream of the pheromone receptor (Wu et al., 1995). The group of Ste20-like kinases is characterized by the presence of a signature sequence 'GTPY/FWAPE' (Dan et al., 2001). A subgroup of these kinases is defined by the presence of a p21 GTPase-binding motif and therefore broadly classified as 'p21-activated kinase' (PAK) family

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(Hofmann et al., 2004). However, a larger number among the Ste20-like kinases does not harbor any well-defined regulatory module and belongs to the germinal center kinase (GCK) family (Kyriakis, 1999). In addition, the topology of the kinases differs as well: PAKs harbor a C-terminal catalytic domain whereas the GCKs usually have their catalytic domain at the N-terminus. The increasing number of sequenced genomes allowed a fine tuning of phylogenetic relationships and further classification of Ste20-like kinases into eight different subfamilies based on conserved protein stretches within and outside the catalytic domain (Dan et al., 2001).

Recent data suggest that the Ste20-like kinases control key reactions in cell division, cell growth, cell polarity, apoptosis and reorganization of the cytoskeleton. With the identification of Ste20 as a MAP4 K, the role of other Ste20-like kinases in activating signaling pathways is not surprising (Drogen et al., 2000; Edmunds and Mahadevan, 2004; Wu et al., 1995). Following the phylogenetic analysis of Dan et al. (2001), kinases belonging to the GCK-I, -IV and -V subfamilies apparently regulate the JNK pathway, whereas members of the GCK-VI and -VIII subfamilies activate the p38 kinase cascade. MST1 and MST2 from the GCK-II subfamily were also shown to be part of MAPK pathways (O'Neill et al., 2005; Watabe et al., 2000).

Dictyostelium discoideum, a genetically tractable haploid organism, is a well-suited model system to investigate the regulation of the cytoskeleton (Noegel and Schleicher, 2000) and the development from single cells to a multicellular organism (Chisholm and Firtel, 2004). The recently completed sequencing of the genome (Eichinger et al., 2005) helped to reveal four PAKs (Chung and Firtel, 1999; Lee et al., 2004; Müller-Taubenberger et al., 2002) and thirteen highly homologous GCKs (our own data). We previously purified the first *D. discoideum* GCK homolog based on its ability to phosphorylate the F-actin-fragmenting protein severin in vitro (Eichinger et al., 1998). Here, we report cloning of *krsA* and characterization of its product Krs1 (kinases responsive to stress), a second member of the GCK family. Phylogenetic analysis groups this kinase into the GCK-II subfamily along with mammalian MST1/Krs2, MST2/Krs1 (Creasy and Chernoff, 1995a, b; Taylor et al., 1996) and the *Drosophila* homolog Hippo (Harvey et al., 2003; Wu et al., 2003). This is in good agreement with a recent thorough *D. discoideum* kinome analysis which lists Krs1 in the MST subfamily (Goldberg et al., 2006). Similar to its homologs, Krs1 possesses a dimerization motif and an autoinhibitory consensus region in its regulatory domain. Despite a peculiar regulation of *krsA* transcription during development, phenotypic changes in the Krs1 null strains were subtle. Expression of a GFP fusion protein suggests a role of Krs1 at the cell membrane and during multicellular motility.

Materials and methods

Cell culture, transformation and development

Wild-type AX2 were grown in HL5 medium and transformed by electroporation as described previously (Faix et al., 2004). Knockout or overexpressing mutants were grown in HL5 supplemented with either blasticidin (10 μg/ml) or neomycin (20 μg/ml). For development in submerged culture, cells were washed and resuspended in 17 mM phosphate buffer, pH 6.0, at a density of 5×10^6 cells/ml, and starved for 6 h. Formation of streams was monitored by light microscopy using a Zeiss Axiovert 40 CFL. For development on solid substratum, growing cells were harvested, washed and plated on phosphate agar at a density of 1×10^8 cells per 10-cm plate.

cDNA, generation of transformation vectors and protein expression

The *krsA* cDNA was amplified from RNA isolated from vegetative cells, cloned into a pUC18 vector and sequence verified. To express the kinase as a GFP fusion, Krs1 was cloned into pDGFP-MCS-Neo (Dumontier et al., 2000) under the control of the act15 promoter. The knockout construct in the pLPBLP vector was essentially done as described previously using a gene replacement approach (Faix et al., 2004). The 5′ and 3′ fragments were amplified from genomic DNA with the following primers: 5′CCCATCGATG AGAA ATTG GGTGAAGGTTCATATGGTTC-3′, 5′-CCCAAGCTTTCTTGATGGATTTGGAATCATAA-ATATG-3′ and 5′-ACCACCACCAAAATTAACAGA-ACCA-3′, 5′-CGCGGATCCGATTTCTTTTCATCAAT-TAA-3′. The 5′ fragment was cloned into the ClaI/HindIII sites; the 3′ fragment into the PstI/BamHI sites of the pLPBLP vector. The vector was digested with BamHI and ClaI before transformation. Individual clones were screened by immunoblotting using the antiserum SA7653 raised against the MBP-Krs1 regulatory domain.

Full-length Krs1 and various C-terminal deletion fragments were cloned into the pMAL-c2X plasmid (New England BioLabs) using the BamHI and PstI sites. Routinely, bacteria were grown at 37 °C and induced at an OD_{600} of 0.6 with 1 mM IPTG for 3 h. Purification of recombinant protein was done according to standard procedures.

Phosphorylation assays

Kinase assays were done as described previously (Eichinger et al., 1998). MBP-Krs1 activity was assayed in a reaction mixture (40 μl) containing 10 mM Tris/