FEBS Letters 588 (2014) 1899–1905

journal homepage: www.FEBSLetters.org

The *Schizosaccharomyces pombe* Hikeshi/Opi10 protein has similar biochemical functions to its human homolog but acts in different physiological contexts

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ARTICLE INFO

Article history:
Received 19 March 2014
Revised 11 April 2014
Accepted 14 April 2014
Available online 24 April 2014

Edited by Francesc Posas

Keywords:
Hikeshi
Opi10
Hsp70
Heat shock
Nuclear transport
Schizosaccharomyces pombe

ABSTRACT

Human Hikeshi (*Hs*Hikeshi) is a nuclear import carrier for Hsp70s and is required for cell survival after heat shock. The Hikeshi homolog in *Schizosaccharomyces pombe* (*Sp*Hikeshi/Opi10) localizes to the nuclear rim, interacts with the Hsp70 homolog Ssa2, and mediates its nuclear import in a reconstituted mammalian nuclear transport system. However, *Sp*Hikeshi/Opi10 is not required for heat stress response and survival after heat stress. Instead, *Sp*Hikeshi/Opi10 is required for the normal expression of stress response genes under optimal conditions and for cell growth during glucose deprivation. Here, the functions of *Sp*Hikeshi/Opi10 are discussed and compared to the functions of *Hs*Hikeshi.

Structured summary of protein interactions:
SpHikeshi/Opi10 physically interacts with **Ssa1** by anti tag coimmunoprecipitation (View interaction)
SpHikeshi/Opi10 physically interacts with **Ssa2** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

Most nuclear proteins are transported between the cytoplasm and the nucleus by nucleocytoplasmic transport receptors (NTRs) of importin-β family (Imp-βs) through nuclear pores embedded in the nuclear envelope [1]. The transport by Imp-βs depends on the gradient of the GTP- and GDP-bound forms of the small GTPase Ran across the nuclear envelope [2]. Importin-α family proteins (Imp-αs) are adaptors for importin-β, the most well studied member of the Imp-β family [3]. In mammalian cells under heat stress conditions, these predominant importin-mediated transport pathways are down-regulated because of the collapse of the RanGTP/RanGDP gradient and the nuclear retention of Imp-αs [4,5]. In mammalian and *Drosophila* cells, however, Hsp70s relocate from the cytoplasm to the nucleus during heat stress [6–9]. *Hs*Hikeshi is a nuclear transport carrier of Hsp70s and facilitates the nuclear import of Hsp70s under heat stress conditions. The nuclear import of Hsp70s by *Hs*Hikeshi is crucial for the attenuation and reversal of the heat-shock response and, thus, for cell viability after heat shock [10]. Although the gene coding for *Hs*Hikeshi (*C11orf73*) is conserved among eukaryotes, including *Schizosaccharomyces pombe* (SPBC21H7.06c) and *Saccharomyces cerevisiae* (*YOL032W*), no thermo sensitive mutants of the homologs have been described in yeasts. The only reported homolog is the *S. cerevisiae OPI10* gene, and the *opi10* mutant is associated with the Opi$^-$ phenotype: overproduction and excretion of inositol in the absence of inositol and choline [11]. However, the biochemical functions of both *S. cerevisiae* Opi10p (*Sc*Opi10p) and *Sp*Hikeshi/Opi10 have yet to be characterized. Thus, the *S. pombe* gene is tentatively annotated as an "*OPI10* homolog" due to sequence identity. Here, we characterized *Sp*Hikeshi/Opi10 to explore the functional conservation between the human and *S. pombe* homologs.

Abbreviations: EMM, Edinburgh minimal medium; GFP, green fluorescent protein; Imp-αs, importin-α family proteins; Imp-βs, importin-β family proteins; MAPK, mitogen activated protein kinase; NPC, nuclear pore complex; NTR, nucleocytoplasmic transport receptor; SAPK, stress activated protein kinase; YE, yeast extract medium; YFP, yellow fluorescent protein

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http://dx.doi.org/10.1016/j.febslet.2014.04.018
0014-5793/

Table 1
S. pombe strains.

Strain	Genotype	Reference or source
ED666	*h⁺ ade6-M210 ura4-D18 leu1-32*	Bioneer Inc.
ED666 (*opi10Δ*)	*h⁺ opi10::kanMX6 ade6-M210 ura4-D18 leu1-32*	Bioneer Inc.
AM2 (*opi10-YFP*)[a]	h^{90} *leu1-32::opi10*^{YFH}*-leu1⁺*	[17]
AM2 (*ssa2-YFP*)[a]	h^{90} *leu1-32::ssa2*^{YFH}*-leu1⁺*	[17]
AM2 (*ssa2-YFP opi10Δ*)[a]	*h⁺ opi10::kanMX6 leu1-32::ssa2*^{YFH}*-leu1⁺*	This work[b]

[a] YFP is contained in a C-terminal YFP-FLAG-His_6 (YFH) tag.
[b] Constructed by genetic crosses as described [12].

2. Materials and methods

2.1. Strains

The *S. pombe* strains used are listed in Table 1. Yeast extract (YE) medium and Edinburgh minimal medium (EMM) [12] were supplemented with adenine, uracil, and leucine as needed. The disruption of the *opi10* gene and the absence of the *Sp*Hikeshi/Opi10 protein in the strain ED666 (*opi10Δ*) (Bioneer Inc.) were confirmed by PCR and Western blotting, respectively (Supplementary Fig. S1).

2.2. Plasmids and proteins

The cDNAs coding for *Sp*Hikeshi/Opi10 and Ssa2 (SPCC1739.13) were provided by the National BioResource Project of Japan (clone names: spa73g12 and spa101a06, respectively). To express *Sp*Hikeshi/Opi10 in *S. pombe*, the cDNA was inserted into the pREP81 vector [13] with and without an N-terminal FLAG tag sequence. The *Sp*Hikeshi/Opi10-His_6 protein was expressed in *Escherichia coli* using the pET21b vector (Novagen), purified on Ni-NTA agarose (Qiagen), and passed through HiTrap Q and Mono S columns (GE healthcare) for further purification. The His_6 tagged green fluorescent protein (GFP)-Ssa2 fusion protein was expressed using the pQE80L vector (Qiagen) in *E. coli* and purified on TALON resin (Clontech).

2.3. FLAG-affinity purification and mass spectrometry

ED666 (*opi10Δ*)(pREP81-*opi10*) and ED666 (*opi10Δ*)(pREP81-*FLAG-opi10*) were grown in EMM at 30 °C to mid-log phase (3×10^7 cells/mL) and harvested. The cells were frozen in liquid N_2, disrupted by Cryopress (Microtech Nichion), and suspended in ×1 cell volume of ×2 concentration of Nonidet buffer (NB) (20 mM HEPES-KOH (pH 7.3), 110 mM KOAc, 2 mM MgOAc, 5 mM NaOAc, 0.5 mM EGTA, 2 mM dithiothreitol, 0.2% Nonidet P40 and 1 μg/mL of each aprotinin, pepstatin A, and leupeptin). The lysates were centrifuged at 200,000×*g* for 15 min, and ×1/50 bed volume of anti FLAG M_2 agarose (Sigma) equilibrated with NB was added to the supernatants. The mixtures were rotated for 2.5 h, and the resin was washed three times with NB. Then, the proteins were eluted with 100 μg/mL of FLAG peptide in NB and separated by SDS–PAGE. The protein band was excised and in-gel-digested using trypsin. The peptides were analyzed by LC–MS/MS (LTQ, Thermo Fisher Scientific) and identified using Mascot (Matrix Science) against the NCBI database. The parameters and the results of the Mascot search are shown in Supplementary Table S1.

2.4. Transport assay

Digitonin-permeabilized HeLa S3 cells were prepared [14] and a transport reaction was reconstituted using a heat-shocked HeLa S3 cytosolic extract depleted of Imp-βs and *Hs*Hikeshi as described previously [10].

2.5. Live-cell imaging of the S. pombe cells

S. pombe cells were cultured in a glass-bottom dish coated with concanavalin A in liquid EMM, and fluorescent images were captured with a DeltaVision RT microscopy system (Sekitechnotron). The temperature was quickly shifted up (to 43 °C) or down (to 30 °C) by medium exchange, and controlled by a heat chamber (MI-IBC Olympus). The medium temperature was directly measured by a probe thermometer (HD1100K Anritsu).

2.6. DNA microarray

ED666 and ED666 (*opi10Δ*) were cultured in liquid EMM at 30 °C. When the cell density was 3×10^7 cells/mL, the temperature was shifted to 43 °C for 1 h and then shifted back to 30 °C. Cells were harvested at four time points: at 30 °C before the initial shift up of temperature (t_1), after 1 h at 43 °C (t_2), and 1 (t_3) and 3 h (t_4) after the shift back down to 30 °C. The cells were disrupted with glass beads and RNA was purified as described [12,15]. cDNA was synthesized and labeled with biotin using a GeneChip 3'IVT Express kit (Affymetrix) and hybridized to GeneChip Yeast Genome 2.0 arrays (Affymetrix). After incubation with streptavidin phycoerythrin, the arrays were scanned using a GeneChip Scanner 3000 7G (Affymetrix). The data were analyzed with GeneSpring GX ver. 12.5 (Agilent Technologies) with 75th percentile normalization. The experimental condition and data were deposited to the Gene Expression Omnibus database with the accession number GSE52259.

3. Results

3.1. SpHikeshi/Opi10 interacts with the Hsp70 homolog Ssa2 in S. pombe and mediates its nuclear import in a mammalian transport system

The gene coding for *Hs*Hikeshi is conserved in *S. pombe* and *S. cerevisiae*. Fig. 1 shows the deduced amino-acid sequences aligned by Clustal W2 [16]. In this alignment, 83, 38, and 53 amino acids are identical between *Sp*Hikeshi/Opi10 and *Hs*Hikeshi, *Sc*Opi10p and *Hs*Hikeshi, and *Sp*Hikeshi/Opi10 and *Sc*Opi10p, respectively (the protein lengths are shown in Fig. 1). *Sp*Hikeshi/Opi10 is more similar to *Hs*Hikeshi (42% identity) than *Sc*Opi10p (19% identity to human), suggesting that the function of *Sp*Hikeshi/Opi10 may be more similar to that of *Hs*Hikeshi.

When an *Sp*Hikeshi/Opi10 protein fused to yellow fluorescent protein (YFP) (*Sp*Hikeshi/Opi10-YFP) was expressed in *S. pombe*, it localized mainly to the nuclear envelope in a punctate manner reminiscent of nuclear pore complexes (NPCs) (as in the *S. pombe* Postgenome database [17]) (Fig. 2A). Thus, *Sp*Hikeshi/Opi10 possibly interacts with the NPCs like *Hs*Hikeshi. To identify *S. pombe* soluble proteins that directly interact with *Sp*Hikeshi/Opi10, we expressed FLAG tagged or untagged *Sp*Hikeshi/Opi10 in the *S. pombe opi10Δ* strain and isolated protein complexes from the cell extracts using an anti-FLAG antibody. One protein with the molecular mass of 70 kDa specifically associated with the *Sp*Hikeshi/Opi10 (Fig. 2B). LC–MS/MS analysis revealed that the protein was Ssa2, an Hsp70 homolog (Supplementary Table S1). Ssa1, another Hsp70 homolog, was also identified, though it had lower peptide coverage. In the control (untagged) lane in Fig. 2B, a faint band with the same mobility as Ssa2 is seen, but the level is negligible. To determine whether *Sp*Hikeshi/Opi10 can import Ssa2 into cell nuclei, purified *Sp*Hikeshi/Opi10 and GFP-Ssa2 were analyzed in