Interaction between the fission yeast nim1/cdr1 protein kinase and a dynamin-related protein

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Abstract The nim1/cdr1 protein kinase is required for an efficient adaptation of cell cycle parameters to changes in nutritional conditions. We have isolated msp1, a new fission yeast member of the dynamin-related large GTPase family, in a two-hybrid screen designed to identify proteins interacting with the nim1 kinase. Msp1 has been shown to be essential for the maintenance of mtDNA and hence for the inheritance of functional mitochondria. We present evidence indicating that nim1 and msp1 proteins physically interact both in vitro and in vivo in fission yeast. These interactions occur through the amino-terminal catalytic domain of nim1 and the carboxy-terminal putative regulatory domain of msp1. These results provide new evidence for the existence of a connection between mitochondrial function and the cell cycle machinery.

Key words: nim1/cdr1; Dynamin; Fission yeast; Cell cycle; Mitochondrial DNA

1. Introduction

The eukaryotic cell cycle is regulated by a family of cyclin-dependent kinases, whose well known head of file is the p34cdc2 gene product initially identified in the fission yeast *Schizosaccharomyces pombe* [1]. The catalytic activity of these enzymes is regulated through the association with cyclin regulatory subunits and through a balance of phosphorylation and dephosphorylation events [2]. The wee1 tyrosine kinase phosphorylates cdc2, while the cdc25 tyrosine phosphatase dephosphorylates and activates it at mitosis. In fission yeast, the nim1 (new inducer of mitosis) kinase is a negative regulator of wee1 [3,4]. Overexpression of nim1 leads to a premature entry into mitosis that is correlated to the activation of cdc2. Conversely, as a consequence of an increased wee1 activity on cdc2, nim1 gene disruption delays G2/M transition. The nim1 kinase was also identified under the name of cdr1 (changed division response 1), as an actor of the cellular response of fission yeast cells to nutritional signals [5–8].

To obtain new insight into the function of nim1 and to identify potential regulators, a yeast two-hybrid protein interaction screening was undertaken to identify proteins that interact with the fission yeast nim1 kinase. Using a similar strategy Wu and Russell recently identified a novel mitotic inhibitor called Nif1 that interacts with the catalytic domain of nim1 [9]. However, the function of Nif1 and the physiological properties of the Nif1-nim1 interaction still remain unknown. In our study, one interacting clone was found to encode msp1, the fission yeast homologue of the *Saccharomyces cerevisiae* MGM1 dynamin-related protein. As we reported elsewhere, *msp1*$^+$ is an essential gene whose conditional loss of function affects the maintenance of mitochondrial DNA and leads to growth arrest associated with respiratory deficiency [10]. Dynamins are large GTPases sharing in their amino-terminal half consensus domains for GTP binding and hydrolysis and a 'dynamin' signature, while they diverge in their long carboxy-terminal extension. The most extensively studied member of this family, the brain-specific isoform dynamin I [11,12], has been shown to play an essential role in clathrin-dependent endocytosis [13] and in the internalization of caveolae in mammalian cells [14,15]. Dynamin-related proteins have been identified in a wide range of organisms and are playing various functions that are not yet fully understood (see [16] for review).

The interaction reported here, between a protein that plays a role in mitochondrial DNA maintenance and a cell cycle regulator required for the adaptation to changes in nutritional conditions, is the first molecular evidence for a link between the cell cycle machinery and an essential cellular function, i.e. mitochondrial biogenesis.

2. Materials and methods

2.1. Yeast strains, cultures

Fission yeast and budding yeast growth media were from Bio 101 (La Jolla, CA). *S. pombe* strain SP199 (h^{+n} *leu1-32 ura4D18 ade6-216*) was grown on medium lacking either leucine (pREP1-nim1HA selection) or uracil (pREP42-msp1 selection) depending on the construct. pREP plasmids carry the *nmt1* repressible promoter and expression was kept under repressed conditions by addition of 4 μM thiamine to liquid media [17,18]. Derepressed conditions were obtained by washing the cells three times in minimal medium and growing them in the absence of thiamine. Budding yeast strains SFY526 (*MAT*a, *ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,112*, *can*^{r}, *gal4-542, gal80-538, URA3::GAL1-lacZ*) [19] and HF7C (*MAT*a, *ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::GAL1-LacZ*) were used for the two-hybrid assays.

2.2. Two-hybrid screen and interaction studies

Bait plasmids were constructed by cloning the nim1 coding sequences in frame with the GAL4 DNA binding domain of the pGBT9 cloning vector (Clontech). Prey plasmids were constructed in the pGAD vector by inserting the wee1 coding sequence in frame with the GAL4 DNA activating domain. The *S. pombe* cDNA library used in this study was constructed in the pGAD vector (Clontech). The screen was performed according to the instructions of the manufacturer in the presence of 50 mM 3-aminotriazole (3AT). In the interaction assay reported in Fig. 1, 2×10^4 HF7C transformed cells were inoculated either on plasmid-selective (without Leu, Trp) or on interaction-selective (without His, with 10 mM 3AT) media. Growth was examined after 4 days at 30°C. The SFY strain was used for the filter β-galactosidase assay reported in Table 1. Cells were grown on selective plates, then transferred on Whatman paper and subjected to six freezing-thawing cycles on liquid nitrogen. The filters were incubated in buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl,

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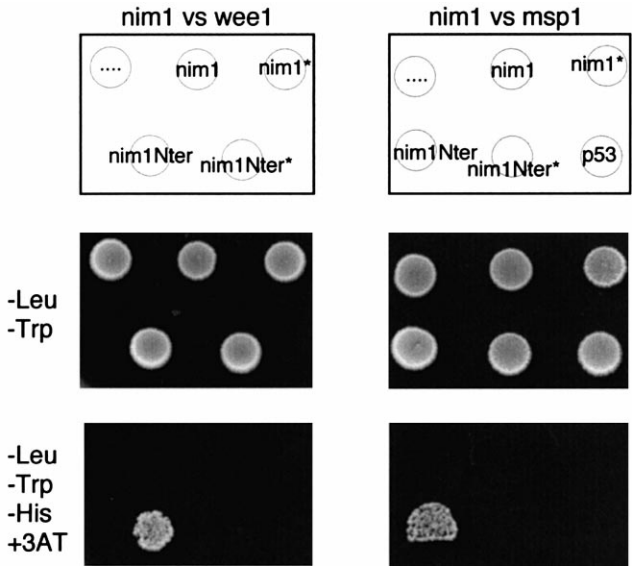

Fig. 1. Interaction between nim1, wee1 and msp1 in the two-hybrid assay. Two-hybrid assay of the nim1 interaction with wee1 and the msp1 clone isolated in the two-hybrid screen (residues 477–903) using the indicated constructs: nim1, truncated nim1 (nim1Nter, residues 1–354) and catalytically inactive forms (nim* and nim1Nter*: substitution of lysine 41 to alanine). Nim1 constructs were in fusion with the GAL4 binding domain (bd). The msp1 and the wee1 constructs were in fusion with the GAL4 DNA activating domain (ad). Transformed cells were inoculated on plasmid-selective media (−Leu, −Trp) with or without selection for the interaction (−His, +10 mM 3AT).

1 mM $MgSO_4$, 40 mM β-mercaptoethanol, pH 7.0) in the presence of 0.35 mg/ml X-gal at 30°C for up to 30 h and the relative coloration (turning blue) was monitored.

2.3. In vitro interaction

Full length msp1 and carboxy-terminal domain (residues 477–903) were cloned in the pGEX vector in frame with GST, then produced in *Escherichia coli* and purified on glutathione Sepharose beads according to [20]. Yeast cells were harvested by centrifugation then broken in the presence of an equal amount of cold 500 μm glass beads in LB lysis buffer (50 mM Tris pH 7.4, 250 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM DTT and 0.1% Triton X-100) containing the following protease inhibitors: 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, 5 μg/ml TLCK (tosyl L-lysine chloromethyl ketone) and 10 μg/ml TPCK (tosyl phenylalanine chloromethyl ketone). The samples were vortexed until approximately 90% of the cells were disrupted. The soluble protein fraction was recovered by three centrifugations of 5 min at 19 000 × *g*. Fusion GST-msp1 proteins loaded on glutathione Sepharose beads were incubated in the presence of 1 mg yeast cellular extracts for 6 h at 4°C on rotator, then washed three times in LB. Samples were boiled for 3 min in Laemmli sample buffer and electrophoresed on 12% SDS-polyacrylamide gel [21]. Electrotransfer onto Hybond C membrane was performed as described [22]. Immunodetections with anti-HA monoclonal antibody (12CA5, Boehringer) were performed with the Renaissance chemiluminescence detection kit (NEN).

2.4. In vivo interaction

Extracts of fission yeast strains overexpressing either HA epitope-tagged nim1, or msp1, or both proteins were prepared as described above except that 0.1% SDS, 0.5% DOC and 1% NP40 were added to the LB. After a five-fold dilution in LB, immunoprecipitations were performed with 2 mg of proteins using monoclonal anti-HA antibody or polyclonal anti-msp1 antibodies [10] for 3 h on a rotator at 4°C. Protein A Sepharose was then added and after a further 1 h incubation the precipitates were washed three times in LB and processed for Western blot with anti-HA antibody as above.

3. Results

3.1. Isolation of nim1 binding proteins

In order to identify proteins that physically interact with the fission yeast nim1 kinase a two-hybrid screening was undertaken. Preliminary experiments were performed between nim1 and its known substrate, wee1 kinase. As shown in Fig. 1, only the N-terminal active catalytic domain of nim1 (nim1Nter, residues 1–354) was found to be able to interact tightly with the wee1 protein when the histidine reporter gene was used. A weaker interaction was detected with full length nim1 and with the catalytically inactive forms (K41A substitution) when the β-galactosidase activity was monitored (Table 1). This discrepancy reflects differences in the expression levels of the fusion proteins, full length nim1 being very poorly expressed (data not shown). The catalytic domain of nim1 was therefore subsequently used to search for interactors in a *S. pombe* cDNA library. From a screen of 4.5×10^6 independent clones, one potential candidate was repetitively isolated. This 1167 bp partial cDNA encoded a 373 residue open reading frame fused to the GAL4 transcriptional activator domain. Cloning of the full length sequence was achieved using a *S. pombe* ordered genomic library provided by the Reference Library DataBase (RLDB). As we reported elsewhere [10], this gene is essential and encodes a new fission yeast dynamin-related protein that is homologous to the budding yeast MGM1 (mitochondrial genome maintenance 1) gene product.

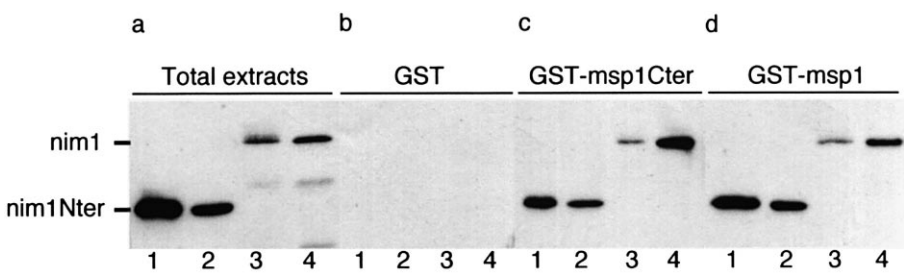

Fig. 2. In vitro interaction between nim1 and msp1. Cell extracts from wild type fission yeast cells expressing HA epitope-tagged nim1Nter (lanes 1), nim1Nter K41A (lanes 2), nim1 (lane 3) and nim1K41A (lanes 4) were used to examine the in vitro interaction with recombinant GST (b), GST-msp1Cter (c), and GST-msp1 (d). Msp1Cter is the nim1-interacting clone identified in our two-hybrid screen, which encodes the carboxy-terminal half of the msp1 protein (residues 477–903). Total cell extracts (a) and proteins bound to the fusion proteins (b–d) were immunoblotted with 12CA5 monoclonal anti-HA antibody.

Table 1
Interaction between nim1, wee1 and msp1 in the two-hybrid assay

Partners[a]	Binding[b]
wee1-bd	–
wee1-nim1	+
wee1-nim1K41A	++
wee1-nim1Nter	++++
wee1-nim1NterK41A	+
msp1-bd	–
msp1-nim1	+
msp1-nim1K41A	+
msp1-nim1Nter	++++
msp1-nim1NterK41A	+
msp1-p53	–

[a]nim1 constructs were in fusion with the GAL4 DNA binding domain (bd), the msp1 and wee1 constructs were in fusion with the GAL4 activating domain (ad). See legend of Fig. 1 for details.
[b]binding was estimated by monitoring the β-galactosidase activity on filter. The number of plus signs represents the relative coloration intensity (see Section 2).

3.2. The msp1 protein interacts physically with nim1 in the two-hybrid system

The physical interaction between nim1 and the isolated clone of msp1 was investigated in more detail in the two-hybrid system using both the histidine (Fig. 1) and the β-galactosidase reporter genes (Table 1). As illustrated, nim1Nter and msp1 interacted strongly in both assays. The association was judged to be specific, since control interactions between the $GAL4^{ad}$-msp1 clone and $GAL4^{bd}$ alone or fused to the human p53 (used as negative control) were not observed. Interaction of the $GAL4^{ad}$-msp1 clone with full length nim1 protein fused to $GAL4^{bd}$ was found to be weak using the β-galactosidase assay (Table 1) and was not detected when the histidine reporter gene was used (Fig. 1). A similar observation was made when catalytically inactive versions of nim1 were used (Fig. 1 and Table 1). As discussed above, these observations likely reflect the differences in the expression level of the nim1 proteins.

3.3. Msp1 associates with nim1 in vitro and in vivo

We first used an in vitro binding assay to confirm the msp1 interaction with nim1. Recombinant full length and truncated msp1 proteins fused to glutathione *S*-transferase were expressed in *E. coli*. HA epitope-tagged versions of the full length nim1 protein kinase or its catalytic domain (nim1Nter) were expressed in fission yeast under the control of a nmt1 thiamine regulated promoter [7] (Fig. 2a). Dead kinase (K41A mutant) versions of these constructs were also assayed. GST-msp1 fusion proteins were purified on glutathione Sepharose beads and then incubated with fission yeast cell extract producing one of the four versions of nim1. The interaction was detected by Western blot after SDS-PAGE of the material retained on the glutathione beads, using monoclonal anti-HA antibodies. Glutathione Sepharose beads loaded with glutathione *S*-transferase alone were used as negative control (Fig. 2b). As shown in Fig. 2, in agreement with the fact that the msp1 protein was isolated as a protein which binds to the catalytic domain of the nim1 kinase, both the catalytic domain and the full length nim1 interacted in vitro with the recombinant GST-msp1 protein (Fig. 2d). In addition, the catalytic activity of nim1 was not required for an efficient interaction in vitro. Since the msp1 clone that was originally identified with the 'two-hybrid' was restricted to its carboxy-terminal half, we examined in vitro the interaction of truncated msp1 protein (477–903) with the four forms of nim1. The interaction between the carboxy-terminal half of msp1 (msp1Cter) and either form of nim1 was also clearly detected (Fig. 2c). These results indicate that the interaction between msp1 and nim1 is specific and involve the N-terminal catalytic domain of nim1 and the carboxy terminal half of the msp1 protein.

Msp1 and nim1 proteins were also found to associate in vivo in *S. pombe*. Fission yeast strains overexpressing either HA epitope-tagged versions of nim1, or msp1, or both proteins were used in co-immunoprecipitation experiments. As shown in Fig. 3A, when both proteins are expressed the nim1 protein is precipitated from fission yeast cell extract with anti-HA and is co-immunoprecipitated with anti-msp1 antibody. Furthermore, when only HA-tagged nim1 was expressed, immunoprecipitation of the endogenous msp1 was sufficient to co-immunoprecipitate nim1. Similar results were obtained when the experiment was performed with cells expressing msp1 together with truncated and/or catalytically inactive forms of nim1. All forms of nim1 were found to associate with msp1 immunoprecipitates (Fig. 3B). Thus, the co-immunoprecipitation of msp1 and nim1 confirms that the two proteins are physically associated in vivo.

4. Discussion

The msp1 protein has been isolated in a yeast two-hybrid screen designed to identify proteins that interact with the fission yeast nim1 kinase. Msp1 has been found to physically

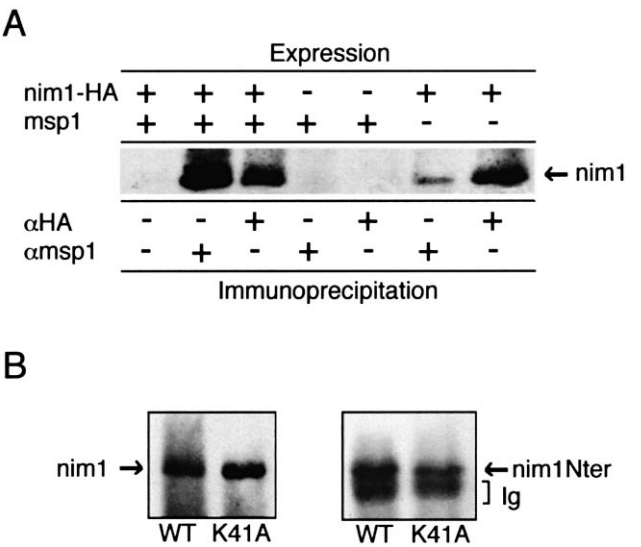

Fig. 3. In vivo interaction between nim1 and msp1. A: Western blot with anti-HA monoclonal antibodies of anti-msp1 or anti-HA immunoprecipitates from extracts of fission yeast cells expressing or not msp1 and a HA-tagged version of nim1 as indicated. The tagged HA-nim1 protein is shown with an arrow. B: Western blot with anti-HA antibodies of anti-msp1 immunoprecipitates from extracts of fission yeast cells. Left panel: cells overexpressing msp1 and HA-tagged versions of full length nim1, either active (WT) or catalytically inactive (K41A). Right panel: cells overexpressing msp1 and HA-tagged version of nim1Nter (WT or K41A). The bracket indicates immunoglobulin chains that are detected by the secondary antibodies.

associate with nim1 both in vitro and in vivo in *S. pombe*. We also found that nim1 interacts in the two-hybrid assay with its known substrate, wee1 kinase. The consequence of the observed interaction between nim1 and msp1 is not understood yet, but this observation raises two types of questions. The first one concerns the characterization of the molecular mechanisms through which nim1 interacts with various regulators, substrates and partners. The second concerns the physiological significance of an interaction between a protein kinase that is involved in cell cycle control and a dynamin-related protein involved in mtDNA maintenance.

Using a similar two-hybrid screen with the nim1 kinase, Wu and Russell recently identified a new mitotic inhibitor called Nif1 that contains a leucine zipper motif but whose function remains unclear [9]. The Nif1-nim1 two-hybrid interaction requires an essential region (amino acids 291–354) that is located just downstream of the catalytic domain of nim1 protein kinase. While a full length nim1 or a protein restricted to amino acids 1–354 fully interacts with Nif1, a nim1 protein that stops at residue 291 does not. The nim1Nter construct that was used in our study and that was shown to interact with msp1 and wee1 is similar and comprises amino acids 1–354. However, we do not know whether these interactions also require the 291–354 region. The observation of an interaction involving the kinase catalytic domain suggested that msp1 could be a substrate of nim1. However, we could not phosphorylate recombinant GST-msp1 using immunopurified nim1 kinase (data not shown). The interaction between nim1 and msp1 may therefore not reflect an enzyme-substrate association, but rather the existence of large multimolecular complexes that may play a role in the connection between cell cycle control and the pathways that regulate mitochondrial biogenesis and function. In that case, the interaction between nim1 and msp1 would be indirect and may be controlled by other proteins which are indeed present in vivo in yeasts and which might also be associated with the cellular nim1 protein used in the in vitro interaction assay.

Several environmental factors such as oxygen level or carbon sources are known to modulate mitochondrial function. This regulation is essential to adjust the energy-producing capability to the cellular needs; however, very little is know about how the signalling pathways that are affected by these factors may interact with nuclear and mitochondrial gene expression (for review see [23]). The nim1 protein has been shown to be required for the efficient cell cycle adaptation to nutritional condition changes [7]. It is also worth mentioning that nim1 shares significant homology with SNF1 [6], a kinase that is required for catabolic derepression of several glucose-repressed genes in budding yeast [24]. Since mitochondrial function is tightly connected with nutritional cues, these observations therefore suggest that the nim1-msp1 interaction may possibly participate in a cross-talk between cell cycle control and mitochondrial biogenesis. Following this line of thought, the interaction between nim1 and msp1 occurs through the C-terminal half of msp1, a domain that has been reported for other dynamins to be required in protein-protein interactions that involved in several signalling pathways [16]. A more precise understanding of both nim1 and msp1 functions is of course now required to examine this hypothesis further.

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