RESEARCH ARTICLE

Characterization of Sro1, a novel stress responsive protein in *Schizosaccharomyces pombe*

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Schizosaccharomyces pombe; stress response; sequence orphan; ROS; PKA; hypothetical protein.

Abstract

The large amount of available genome sequencing data presents a huge challenge in the form of orphan sequences. This study reports the detailed functional characterization of one such orphan sequence in *Schizosaccharomyces pombe*. We identified this gene as a prominently upregulated 1.4 kb transcript in a screen for Cigarette smoke extract responsive genes in *S. pombe* and named it Stress Responsive Orphan 1 (*Sro1*). We report various functions of *Sro1* in regulation of cellular behaviour under stress conditions. We show that this gene (*Sro1*) responds to a variety of stress conditions and that the expression of the gene is regulated mainly through the stress activated protein kinase (SAPK) Sty1 and its downstream transcription factor *Atf1*. Deletion of *Sro1* also significantly alters the reactive oxygen species (ROS) generation profiles and the cell-cycle progression of *S. pombe* during stress conditions. The stress-specific alteration of the ROS generation profiles and checkpoint activation resulting from deletion of the gene suggest that *Sro1* might be a key player in determining cellular responses/fate under stress conditions.

Introduction

Schizosaccharomyces pombe or fission yeast was the sixth eukaryotic organism to have its genome sequenced (Wood *et al.*, 2002). It has a very compact genome, having 13.8 million base pairs distributed among three chromosomes and 4824 genes. Annotation of the sequenced genomes has revealed the presence of a significantly large number of sequence orphans coding for hypothetical proteins (i.e. those which do not bear any sequence homology to any known proteins). Of the 5004 *S. pombe* ORFs currently annotated, only 1607 have experimentally characterized functions. The functions of 2329 genes have been inferred from homology while 364 predicted ORFs have orphan sequences, whose functions evidently remain largely elusive. Studies on the experimental characterization of these sequence orphans could reveal interesting and novel aspects of *S. pombe* physiology and also could facilitate prediction of relevant human orthologues. In many organisms there have been instances when the characterization of novel proteins has actually brought into focus newer aspects of the organisms' life processes (Eisenreich *et al.*, 2001, 2004; Alexandrov *et al.*, 2002; Jackman *et al.*, 2003; Soma *et al.*, 2003). The importance of the experimental investigation of hypothetical protein functions is thus clearly evident.

In this report we present the functional characterization of a hypothetical protein in *S. pombe* (Gene ID: SPBC1347.11) and provide evidence that suggests functional significance for this gene in modulating various essential regulatory

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processes that constitute the stress responses in *S. pombe*. Our laboratory has been engaged in studying cigarette smoke (CS)-mediated cellular stress responses using *S. pombe* as a model system (Chaudhuri *et al.*, 2005) and this particular gene was identified in our laboratory in a screen for CS-responsive genes in *S. pombe* (GenBank Accession Number: AY048753). The ORF sequence of SPBC1347.11 is only 321 bp long, coding for a small 11.4 kDa protein. Whole genome microarray experiments have earlier shown that this particular gene is stress-inducible and is upregulated upon hydrogen peroxide treatment, heat shock and UV irradiation but not in response to cadmium, methyl methane sulphonate (MMS) or sorbitol. Because this gene has been found to be induced under a variety of stress conditions, we have named it Stress Responsive Orphan1 (*Sro1*). Microarray studies have also shown that stress-inducible expression of *Sro1* (SPBC1347.11) is dependent on the *Sty1*/*Spc1* mitogen-activated protein (MAP) kinase and *Atf1* transcription factor (Chen *et al.*, 2003) and on *Rad3* (Watson *et al.*, 2004). Sty1 is the major SAPK in *S. pombe*; *Atf1* is a transcription factor acting downstream of *Sty1* while *Rad3* is the major cell-cycle regulatory kinase in *S. pombe*. Interestingly both these pathways are active in *S. pombe* cells treated with cigarette smoke extract (CSE) (Chaudhuri *et al.*, 2005). Localization of a sro1-yfp fusion protein has been described earlier (Matsuyama *et al.*, 2006), according to which Sro1 protein localizes to mitochondria.

This study mainly investigates the probable function of *Sro1* in *S. pombe* stress response. A distinctive feature of *S. pombe* is that the response to multiple stresses is funnelled through a common integrative pathway through the SAPK *Sty1*. Activation of these SAPK pathways leads to stimulation of specific transcription factors (*Atf*1 and *Pap1*) which then modulate expression of various stress responsive genes. SAPK pathways are also linked to the cell-cycle machinery, and hence their activation leads to growth-arrested condition. This inhibits further damage to the cells and provides some time for it to overcome the stress before entering the growth phase again. In the fission yeast *S. pombe*, the MAPK Sty1 is the effector kinase required for cellular response to a wide range of stimuli including nutritional stress, temperature changes, oxidative status, osmotic conditions, heavy-metal toxicity, and the presence of DNA-damaging agents such as UV light (Shiozaki & Russell, 1995; Kato *et al.*, 1996; Degols & Russell, 1997; Millar, 1999). In this study we report the relationship between the novel protein sro1 and the *S. pombe* stress responses.

Materials and methods

Strains, media and growth conditions

Schizosaccharomyces pombe strains used in this study are listed in Table 1. Cells were grown at 30 °C in the rich medium (YES) as described previously (Moreno *et al.*, 1991). In overexpression experiments Edinburgh Minimal Medium (EMM) lacking leucine was used. For growth on media containing galactose the medium composition was 1% yeast extract, 1% bactopeptone, 0.1% glucose, 2% galactose, and amino acid supplements. For growth on medium containing ethanol the medium used was 1% yeast extract, 3% glycerol, 3% ethanol and amino acid supplements.

Table 1. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype	Source
PR109	*h⁻ leu1-32 ura4-D18*	Paul Russell
KS1366	*h⁻ spc1::ura4⁺ leu1-32 ura4-D18*	K. Shiozaki
TE570	*h⁻ rad3::ura4⁺ ade6-704 leu1-32 ura4-D18*	Tamar Enoch
Δatf1	*h- atf1::ura4 ade6-704 leu1-32 ura4-D18*	K. Shiozaki
Δpap1	*h- pap1::ura4 ade6-704 leu1-32 ura4-D18*	Tamar Enoch
Δchk1	*h- chk1::ura4+ade6-704 leu1-32 ura4-D18*	Tim Humphrey
Δcds1	*h⁻ cds1:: ura4⁺ leu1-32 ura4-D18*	Lab Stock
BG_2493	*h- sro1:: Kan4MX leu 1-32 ura4-D18/h-leu 1-32 ura4-D18*	Bioneer Corporation, Korea

Preparation of aqueous extract of CSE

CSE was prepared as described before (Chaudhuri *et al.*, 2005). Briefly, the smoke trapped from an Indian commercial filtered-tipped cigarette (74 mm) in 50 mM phosphate buffer, pH 7.4, was passed through 0.45 μm filters. After adjusting the pH to 7.4, this filtrate was used as aqueous extract of cigarette smoke (CSE) to treat the cells. One milliliter of filtrate is equivalent to one cigarette.

Cloning of *Sro1*

The *Sro1* gene was amplified from *S. pombe* genomic DNA by PCR using primers 5′-GATTCATCCATATGTTTTCTAT-3′ and 5′-TCGGGCTTAAGCGCTTACTT-3′. *Sro1* was cloned into pBSSK (Stratagene) and subcloned into pGEX4T1 (Amersham Pharmacia) as a glutathione-*S*-transferases (GST) tagged protein. For expression in *S. pombe, Sro1* was subcloned into pREP41EGFPN.

RNA analysis

Total RNA was isolated from untreated or treated yeast cells using SDS/phenol methods as described in the Fission Yeast Handbook. The RNA was resolved on 1.5% 3-(*N*-morpholino) propanesulfonic acid gels, transferred on NYTRAN membranes and probed with ^{32}P labeled Sro1 DNA. Reverse

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transcription was done using M-MuLV RT from MBI according to the manufacturer's protocol. PCR was carried out with primers as described above.

Deletion of *Sro1*

The heterozygous deletion mutant of SPBC1347.11 (BG_2493) was from BIONEER Corp, Korea. This diploid strain was grown on minimal media to allow sporulation and the haploids so isolated were then selected by G418 resistance. The presence of *Sro1* deletion in haploids was further confirmed by PCR using gene-specific primers for SPBC1347.11.

Kinase assays using GST-Sro1

GST-Sro1 was overexpressed in the *Escherichia coli* BL21, recovered from the inclusion bodies, renatured and purified using Glutathione Sepharose CL-4B resin (Amersham Pharmacia). The purified GST-Sro1 was dialyzed against phosphate-buffered saline (PBS) pH 8.0, 1% Triton X-100 and incubated with casein kinase II (CKII) (NEB) or the catalytic subunit of protein kinase A (PKA) (Promega) in the presence of 100 μM ATP and 0.5 μCi of γ-^{32}P ATP (BRIT). The reaction mixture was resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained using Coomassie Blue and then exposed on Storage phosphor screens and scanned using a Typhoon TRIO imager (GE Healthcare).

Cell-cycle studies

For cell-cycle studies, cells were collected at different time intervals, fixed in 70% ethanol, washed with 50 mM sodium citrate, treated with DNAse-free RNAse for 3 h at 37 °C stained with propidium iodide (PI) and then analysed on a Beckton Dickinson FACS Calibur.

Cell viability measurement

Cells were stained with PI without fixing. As only dead cells take up PI, uptake of PI was used to assess cell death. PI uptake was measured by flow cytometry using a Becton Dickinson FACS Calibur. The per cent of cells not taking up PI was used as a measure of cell viability. The data were normalized with respect to untreated cell populations.

In vivo measurement of reactive oxygen species (ROS)

Intracellular oxidation level was monitored using 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA; Sigma). H_2DCF-DA is a nonpolar cell-permeant compound. When it enters the cell, the endogenous esterases convert it to a polar, membrane-impermeant derivative, H_2DCF, which gets trapped within the cell. H_2DCF is nonfluorescent, but is rapidly oxidized to the fluorescent derivative DCF by intracellular H_2O_2 and other peroxides. To measure ROS levels, cells were incubated with 10 μM H_2DCF-DA. DCF fluorescence was then measured by flow cytometry using a Beckton Dickinson FACS Calibur. The per cent of cells having higher fluorescence than the unstained cells was determined as a measure of cell population with elevated intracellular ROS levels.

Polysome profiling in *S. pombe*

Exponentially growing fission yeast cells (8×10^6) were treated with 40 μL CSE for 1 h, then treated with cycloheximide to stop protein synthesis and harvested. Cell extracts (equivalent to 10 $OD_{260\,nm}$ units) were then loaded on 15–45% sucrose gradients and ultracentrifuged at 285 000 ***g*** for 2.5 h. Fractions (0.5 mL) were collected, $A_{254\,nm}$ was measured and plotted to obtain the polysome profile. RNA was also isolated from these fractions and analysed by reverse transcriptase (RT)-PCR with primers specific for *Sro1*.

Microscopy

PR109 cells transformed with pREP41EGFPN-Sro1 were allowed to grow in EMM lacking thiamine at 30 °C for 18 h and then were treated with 2.5 mM H_2O_2 for 1 h. The cells were then incubated with 1 μM Mitotracker Red (Molecular Probes) and then examined under a Zeiss LSM 510 META confocal microscope at × 63 magnification.

Results

Sro1 expression is elevated during various stress conditions

As mentioned earlier Sro1 was identified as a significantly overexpressed transcript present in *S. pombe* cells exposed to CSE. Wild-type *S. pombe* cells were treated with increasing doses of CSE (20 and 40 μL per 8×10^6 cells), total RNA was isolated and Sro1 levels were detected by Northern blotting. This showed that elevation of its expression (7.3-fold for 20 μL CSE and 8.6-fold for 40 μL CSE) occurred in a dose-dependent manner (Fig. 1a). Thereafter, polysomes from control and CSE-treated *S. pombe* cells were isolated and total RNA was extracted from different polysomal fractions. Subsequent RT-PCR analysis revealed that Sro1 transcript was associated with the polysomal fractions of CSE-treated *S. pombe* cells (Fig. 1b). This indicates a possibility that Sro1 may be expressed as a protein or that it may be involved in translational regulation. However, further studies are needed to confirm these observations. In addition to the microarray data available for conditions of Sro1 expression,

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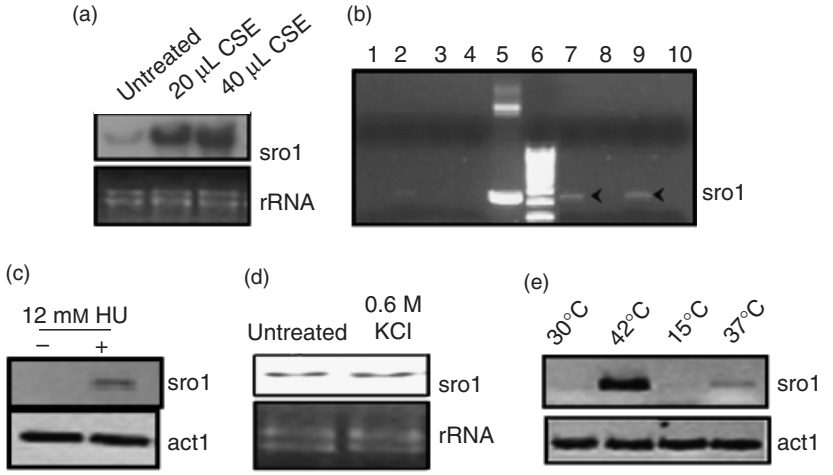

Fig. 1. Conditions of expression of *Sro1*. (a) Wild-type *Schizosaccharomyces pombe* cells were treated with increasing doses of CSE (20 and 40 μL per 8×10^6 cells), RNA was isolated and sro1 transcript levels were determined by Northern blotting. (b) Polysomal fractions of *S. pombe* cells before and after treatment with CSE for 1 h were resolved on 15–45% sucrose gradients and then RNA was isolated from these fractions and checked for the presence of Sro1 transcript. Lanes 1–4, Polysomal fractions from untreated cells; lanes 7–10, Polysomal fractions from CSE-treated cells; lane 5, Positive control for Sro1; lane 6, DNA molecular weight marker. (c) RNA was isolated from *S. pombe* cells treated with 12 mM hydroxyurea and then levels of Sro1 transcript were assayed by RT-PCR. (d) Wild-type *S. pombe* cells were treated 0.6 M KCl, total RNA was isolated and Sro1 levels were detected by Northern blotting. (e) RT-PCR assay was done to investigate expression levels of Sro1 at different temperatures (15, 30, 37 and 42 °C).

we further show that it is upregulated (fourfold) in response to 12 mM hydroxyurea (Fig. 1c). We also show that there is no detectable increase in Sro1 transcript levels during osmotic shock by 0.6 M KCl (Fig. 1d) or during cold shock at 15 °C (Fig. 1e). *Sro1* transcription was found to be upregulated at 37 °C (3.7-fold) and increased much more (12-fold) at 42 °C (Fig. 1e).

Sro1 expression is regulated by the SAPK pathway and by Rad3

Whole genome microarray data have previously indicated that the expression of SPBC1347.11 is dependent on the SAPK *Sty1* and its downstream transcription factor *Atf1* (Chen *et al.*, 2003) in response to various stress stimuli, and also by *Rad3* (Watson *et al.*, 2004) upon exposure to ionizing radiation. We investigated the regulation of expression of *Sro1* during cellular response to CSE treatment and during heat shock. Wild-type, Δ*sty1*, Δ*atf1* and Δ*pap1* cells were treated separately with 40 μL CSE for 60 min and the expression levels of *Sro1* were observed by Northern blot analysis. It was found that *Sty1* and *Atf1* activity was essential for Sro1 expression after CSE treatment (Fig. 2a). Loss of Pap1 on the other hand made very little difference to *Sro1* expression levels (Fig. 2a). *Schizosaccharomyces pombe* wild-type and Δ*sty1* cells were then subjected to heat shock at 42 °C for 60 min, and expression of *Sro1* was monitored through RT-PCR analysis. *Sty1* was once again found to be necessary of induction of *Sro1* expression after heat shock

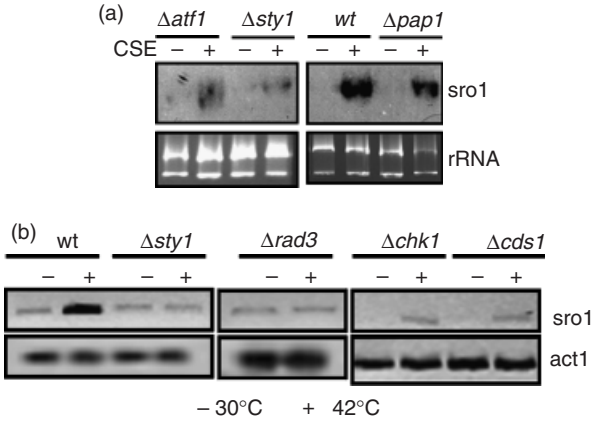

Fig. 2. Regulation of expression of Sro1: (a) Wild-type, Δ*sty1*, Δ*atf1* and Δ*pap1* cells were either treated with 40 μL CSE for 1 h (+) or left untreated (–), total RNA was isolated from these cells, and sro1 levels were detected by Northern blotting. (b) RT-PCR analysis of the RNA isolated from wild-type, Δ*sty1,* Δ*rad3,* Δ *chk1,* Δ*cds1* cells subjected to heat shock at 42 °C (+), or without heat shock (–), reveals that Sty1 and Rad3 are both required for Sro1 upregulation after heat shock.

(Fig. 2b). *Sty1* thus seems to be the common regulatory molecule for *Sro1* expression. Again Δ*rad3*, Δ*chk1* and Δ*cds1* *S. pombe* cells were subjected to heat shock at 42 °C for 60 min and *Sro1* expression levels were analysed by RT-PCR. Surprisingly, it was found that *Rad3* activity was also necessary for induction of *Sro1* expression during heat shock (Fig. 2b). *Chk1* and *Cds1* also seemed to play a role in *Sro1* expression in heat-shocked *S. pombe* cells (Fig. 2b).

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Deletion of *Sro1* reduces the growth rate of *S. pombe* during stress conditions

Δ*sro1* and wild-type *S. pombe* cells were treated with various stress-inducing agents such as 40 μL CSE, 2.5 mM H_2O_2, 0.6 M KCl and 5 mM hydroxyurea and the growth of both the cell populations was monitored at regular time intervals. Deletion of *Sro1* decreased the growth rate of *S. pombe* cells compared with the wild-type cells during stress conditions (Fig. 3a and b). However, overexpression of *Sro1* did not provide the cells with any significant growth advantage (G. Sundaram, S.P. Chaudhari, S. Chaudhari, K. Sheelarani & D. Chattopadhayay, unpublished data). Both Δ*sro1* and wild-type *S. pombe* cells were then treated separately with 40 μL CSE, 2.5 mM H_2O_2, 0.6 M KCl, 5 mM hydroxyurea. They were also subjected to heat shock at 42 °C for 2 h and the cell death in these populations was determined by flow cytometry by the fold increase in PI uptake with respect to untreated controls. We found that the Δ*sro1* cells exhibited a very nominal difference in sensitivity to these stress conditions, as compared with the wild-type population (Fig. 3c).

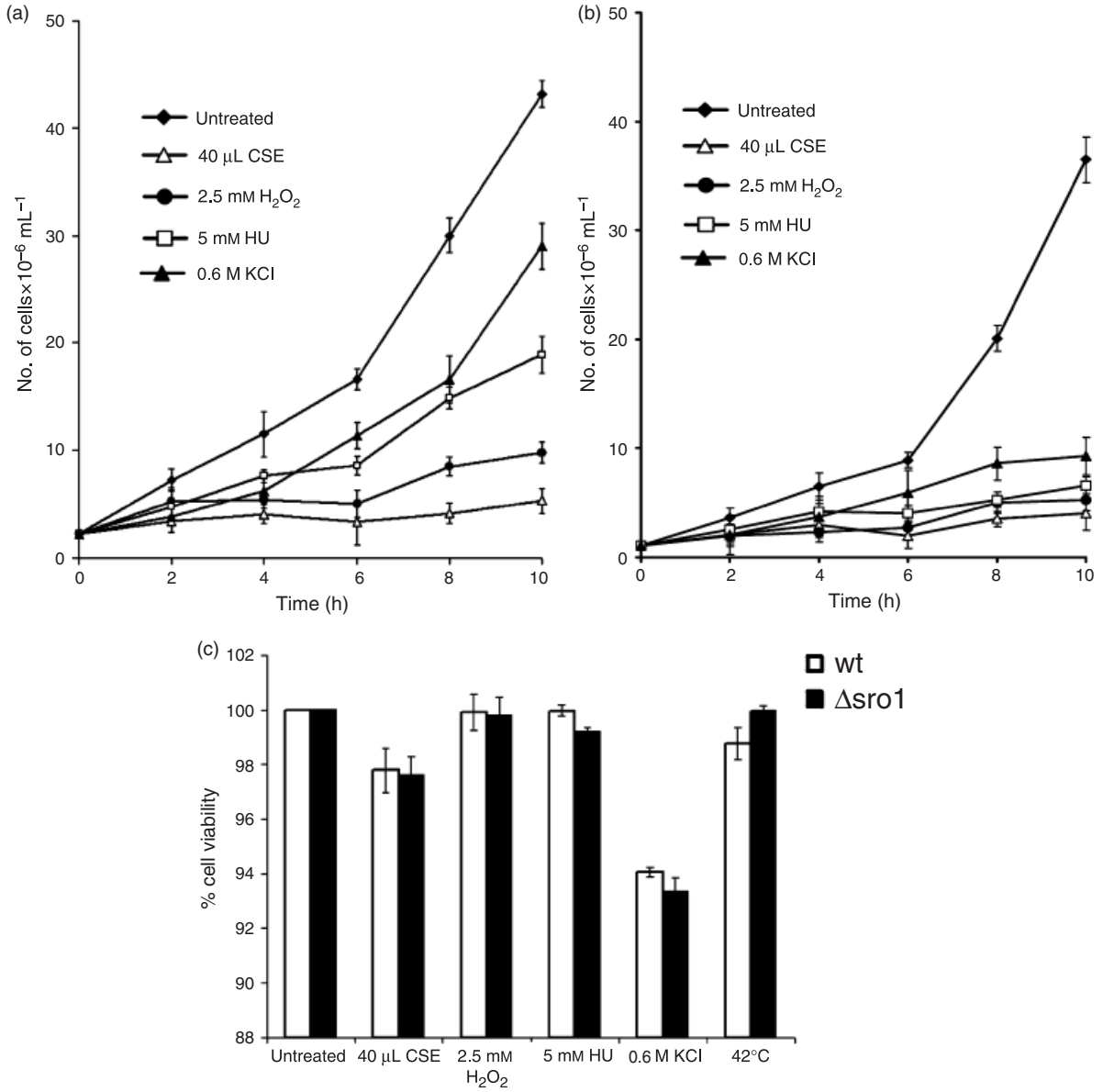

Fig. 3. Δ*sro1* cells have lower growth rates during stress conditions. Growth kinetics of wild-type (a) and Δ*sro1* (b) cells treated with 0.6 M KCl, 2.5 mM H_2O_2, 5 mM hydroxyurea, 40 μL CSE. (d) Viability of wild-type and Δ*sro1* cells subjected to heat shock or treated with 0.6 M KCl, 2.5 mM H_2O_2, 5 mM hydroxyurea, 40 μL CSE. Data represent mean of three independent experiments.

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Deletion of *Sro1* alters the ROS generation profile of stressed *S. pombe* cells

Sro1 has been earlier reported to be a mitochondrial protein. We also found that enhanced green fluorescent protein (EGFP)-Sro1 colocalized with Mitotracker both in untreated and hydrogen peroxide-treated cells (Fig. 4a). Because of its mitochondrial localization and also because Sro1 was identified under stress conditions predominantly oxidative in nature, we tested the possibility of Sro1 having a role in the maintenance of the cellular redox state. Wild-type and Δ*sro1* cells were treated with increasing doses of H_2O_2 and the intracellular ROS levels were monitored at regular time intervals, by staining with the ROS-specific dye H_2-DCFDA (as described in 'Materials and methods'). It was found that the ROS levels in Δ*sro1* cells were always lower than the similarly treated wild-type cells (Fig. 4b). To further investigate the role of Sro1 in the cellular ROS generation pathways, Δ*sro1* and wild-type cells were subjected to heat shock at 42 °C or treated with 40 μL CSE or 5 mM hydroxyurea and the ROS levels were monitored as before. Interestingly, we observed that the temporal ROS generation pattern of Δ*sro1* cells differed from that of the wild-type *S. pombe* cells depending upon the nature of the stress conditions to which the cells were subjected. In CSE-treated cells (Fig. 4c) the initial ROS levels were lower in Δ*sro1* cells. But at later time points, there was a sustained ROS generation in Δ*sro1* cells while the ROS levels in wild-type cells had subsided. In case of treatment with 5 mM hydroxyurea, (Fig. 4d) the initial ROS levels in Δ*sro1* cells were higher than that of wild-type cells but a reverse trend was observed at later time points. In the heat-shocked cells, we found the ROS levels in the Δ*sro1* (Fig. 4e) cells to be consistently lower than that of the wild-type cells. These observations strongly indicate that Sro1 can regulate stress-specific responses of *S. pombe* at least with respect to ROS generation. To further investigate relationship between Sro1 activity and cellular ROS generation, we tested the ability of the Δ*sro1* cells to utilize either galactose or ethanol instead of glucose. Equal number of cells of either type were separately inoculated in media containing glucose, galactose or ethanol and incubated at 30 °C for 16 h. Cell growth was then ascertained my measuring the absorbance of the different cultures at 595 nm. We observed that the growth of both wild-type and Δ*sro1* cells was comparable on media containing either glucose or galactose. Both the cell populations did not grow well on media containing ethanol. However, the growth of Δ*sro1* cells on ethanol-containing media was twofold higher than that of the wild-type cells (Table 2).

Sro1 might be a substrate for PKA

It did not seem very likely that Sro1 could independently affect the intracellular ROS accumulation in *S. pombe*. We hypothesized that *Sro1* could be a part of a signal transduction network that regulates ROS generation/accumulation in *S. pombe*. Computational sequence analysis shows that Sro1 has multiple serine/threonine residues with a high potential for phosphorylation. These sites include those for CKII, cdc2, PKA, protein kinase C and p38 MAPK as predicted by NETPHOSK1.0. One of these kinases namely PKA has been reported to regulate ROS accumulation in *S. pombe* (Roux *et al.*, 2006). We purified a bacterially expressed recombinant GST tagged version of Sro1 and then investigated whether it can be phosphorylated by PKA. We also investigated whether CKII could phosphorylate Sro1 because the former is a key regulatory enzyme involved in many cellular processes, including the control of growth and cell division. We observed that in the case of phosphorylation by CKII a phosphorylated band did appear (Fig. 5) at a molecular weight similar to that of GST-Sro1 (37.1 kDa) but because this band was present even in the control lane for GST phosphorylation, although at a lesser intensity, it was difficult to conclude whether Sro1 can be phosphorylated by CKII. In the case of phosphorylation by PKA, we did observe that GST-Sro1 is actually phosphorylated by PKA, although even in this case there was always a non-specific band with a molecular weight just slightly higher than that of GST-Sro1 (marked by an asterisk in Fig. 5) present in the case of both the GST only and GST-Sro1 samples. Thus, we show that Sro1 may be a substrate for phosphorylation by PKA.

Sro1 also functions in regulation of cell cycle in *S. pombe*

Survival of an organism in stress conditions is largely dependent on its ability to enforce cell-cycle checkpoints which provide the cell with sufficient time to repair the accumulated damage. In order to investigate whether Sro1 could affect the activation of these checkpoints, we separately treated asynchronously growing wild-type and Δ*sro1* cells with 0.6 M KCl and 40 μL CSE and monitored their cell-cycle progression. Significant differences were observed in the cell-cycle progression of similarly treated wild-type (Fig. 6a) and Δ*sro1* cells (Fig. 6b). We have shown earlier CSE treatment activates an S-phase checkpoint in wild-type *S. pombe* cells which accumulate with a DNA content between 1 and 2 N (Chaudhuri *et al.*, 2005). On the other hand, osmotic shock is known to abrogate the G2/M checkpoint in *S. pombe*. We found that 0.6 M KCl-treated wild-type *S. pombe* cells transiently accumulate with a DNA content between 2 and 4 N(for 2 h) but in the case of Δ*sro1* cells, this accumulation continued up to as long as 6 h. Moreover, the KCl-treated Δ*sro1* cells also exhibited the presence of a 'Stretched chromatin' phenotype (Fig. 6c). Hence, Sro1 seems to be essential for recognition/repair of

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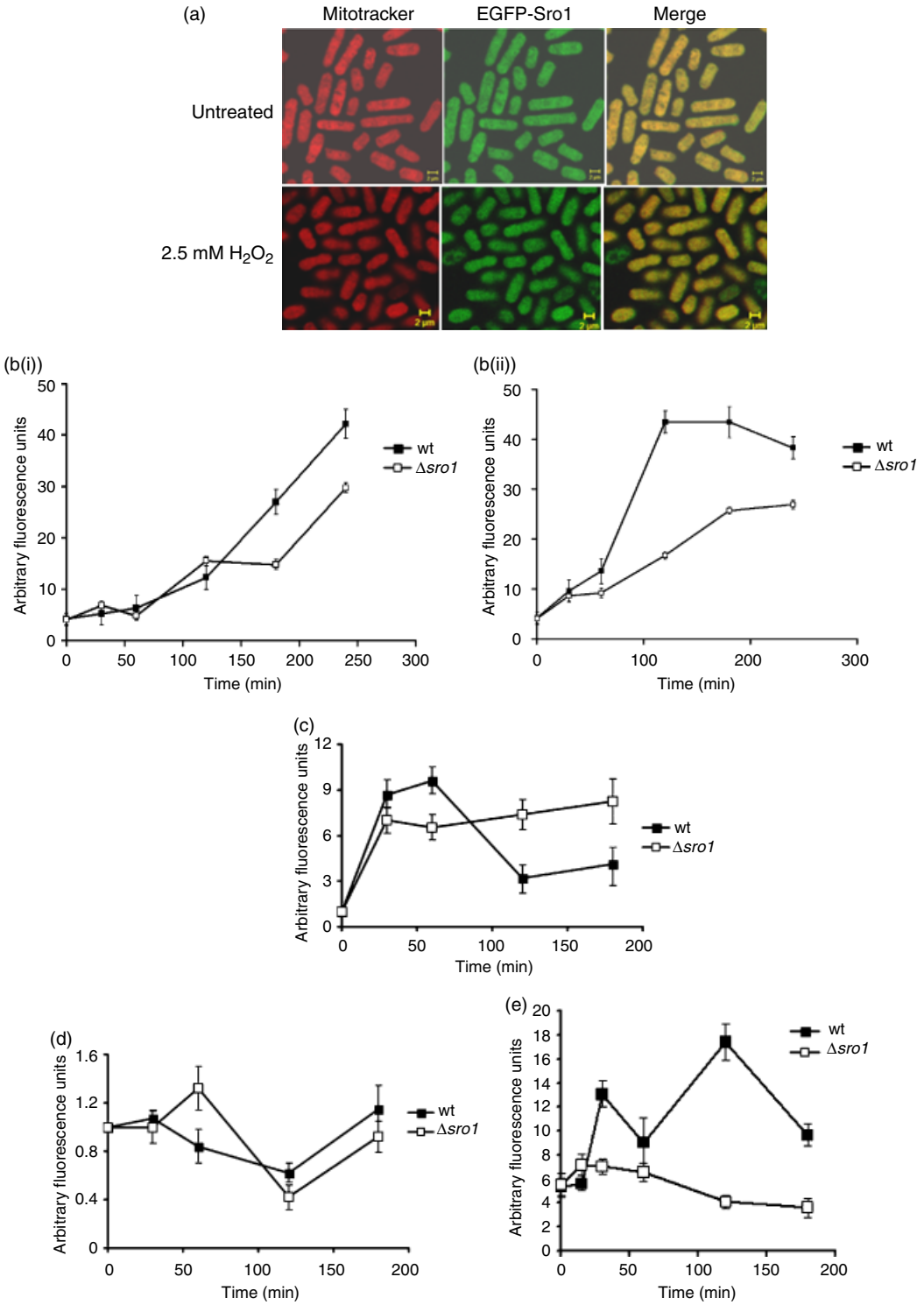

Fig. 4. Sro1 plays a role in cellular ROS generation. (a) Wild-type cells overexpressing EGFP-Sro1 were treated with 2.5 mM H_2O_2, washed, incubated with 1 μM mitotracker and Sro1 was found to colocalize with mitochondria. Wild-type and Δ*sro1* cells were treated with 5 mM H_2O_2 [b (i)], 10 mM H_2O_2 [b (ii)], 40 μL CSE (c), 5 mM hydroxyurea (d) or subjected to heat shock (e) and ROS levels were determined by the DCF fluorescence ('Materials and methods'). Data represent mean of three independent experiments.

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Table 2. Growth in media containing different carbon sources

	$A_{595\,nm}$ after 16 h of growth	
Media containing	Δ*sro1*	wt
2% Glucose	0.650	0.726
3% Galactose 0.1% Glucose	0.207	0.263
3% Ethanol 3%Glycerol	0.032	0.015

Data indicate mean of three independent experiments.

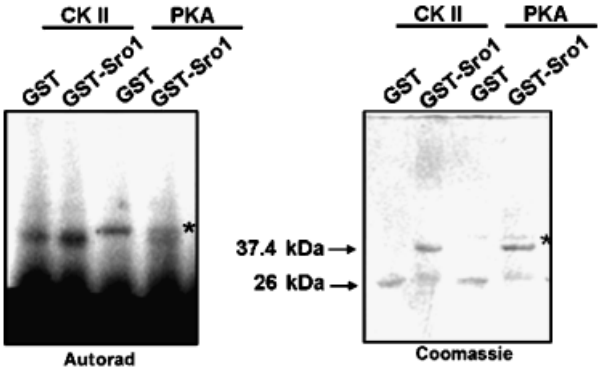

Fig. 5. Sro1 may be a substrate for PKA. Bacterially expressed and purified recombinant GST-Sro1 protein was tested for phosphorylation by the kinases CKII, PKA, in the presence of γ-p32 labelled ATP. Control kinasing reactions with GST alone were performed to eliminate the possibility of the phosphorylation of the GST tag (* indicates a non-specific band).

osmotic shock-induced DNA damage. When both wild-type and Δ*sro1* cells were treated with CSE it was found that activation of the S-phase checkpoint in the latter is delayed. In this case again *Sro1* seems to be playing a similar role. Thus, our observations show that *Sro1* has an effect on the cell-cycle progression of stressed *S. pombe* cells.

Discussion

Investigations of the functions of genes with orphan sequences have the potential to bring into focus many unknown and unexplored aspects of life processes. This study reports the characterization of a novel protein Sro1 in *S. pombe*. Because the sequence of *Sro1* bears no homology whatsoever with any known sequence, it was difficult to get any idea about its probable role in the fission yeast life cycle. To get some information about sro1, sequence analysis was done using bioinformatics software freely available at http://www.expasy.org. Detailed computational sequence analysis revealed a few predictions about the characteristics of the gene and the gene product. For instance, the NETPHOS 2.0 software (Blom *et al.*, 1999, 2004) predicts several serine and threonine residues with a very high phosphorylation potential. The protein also has a potential for *O*-glycosylation as predicted by the YINGOYANG software (Gupta, 2001) The results of Predicted solvent accessibility composition (core/surface ratio) using PROSITE say that the protein is predicted to contain 75.5% residues which are exposed with more than 16% on their surface. However, because the gene has an orphan sequence, the information obtained from sequence analysis was far from sufficient.

The significant upregulation of *Sro1* expression in *S. pombe* cells under a variety of stress conditions clearly indicates an important role for this novel *S. pombe* protein in regulating the response of *S. pombe* cells. This fact is confirmed by our observations, which show that deletion of *Sro1* reduces the growth rate of *S. pombe* cells during stress conditions. The expression of *Sro1* during stress conditions is dependent on both the SAPK pathway as well as the cell-cycle regulatory pathway. The presence of a complex regulatory network determining the expression levels of the protein further indicates that the protein could have multiple functions and that its activity might be necessary for a broad range of cellular processes. The fact that the basal expression levels of the *Sro1* gene are pretty low indicates that either its activity is not essential for normal cellular growth (also corroborated by the fact that its deletion does not significantly affect cellular behavior under standard conditions), or that there could be other genes with overlapping functions. Our results indicate a possibility of Sro1 being phosphorylated by PKA. The latter plays many important roles in the *S. pombe* life cycle and has been implicated in nutrient sensing (Hoffman, 2005) and aging processes (Roux *et al.*, 2006) of *S. pombe*. The fact that *Sro1* may be one of the targets of such an important regulatory protein also reveals that Sro1 itself may potentially play a major role in a subset of processes regulated by PKA. For instance, Δ*pka1* cells are known to exhibit decreased ROS accumulation, a phenotype that is present in stressed Δ*sro1* cells as well. Hence, it is possible that *Sro1* might link *Pka1* to the ROS generation machinery in *S. pombe*. We have shown that deletion of *Sro1* changes the ROS generation profile under various stress conditions in *S. pombe* cells. This indicates an important function for *sro1* in the cellular ROS generation pathway. Interestingly, we observed that there are significant differences in the temporal ROS generation profiles of wild-type and Δ*sro1* cells, and that the nature of these differences depends upon the nature of the stress to which the cells were exposed. Our results show that *sro1* can function in both the elevation of ROS levels as well as lead to the scavenging of the ROS in a stress-dependent manner. Deletion of *sro1* made the cells more efficient in utilizing glycerol and ethanol as a carbon source, indicating that the Sro1 protein may somehow be associated with cellular respiration. In accordance with the regulation of *sro1* expression by the cell-cycle regulatory kinase *Rad3*, we show that loss of *sro1* significantly changes the time or

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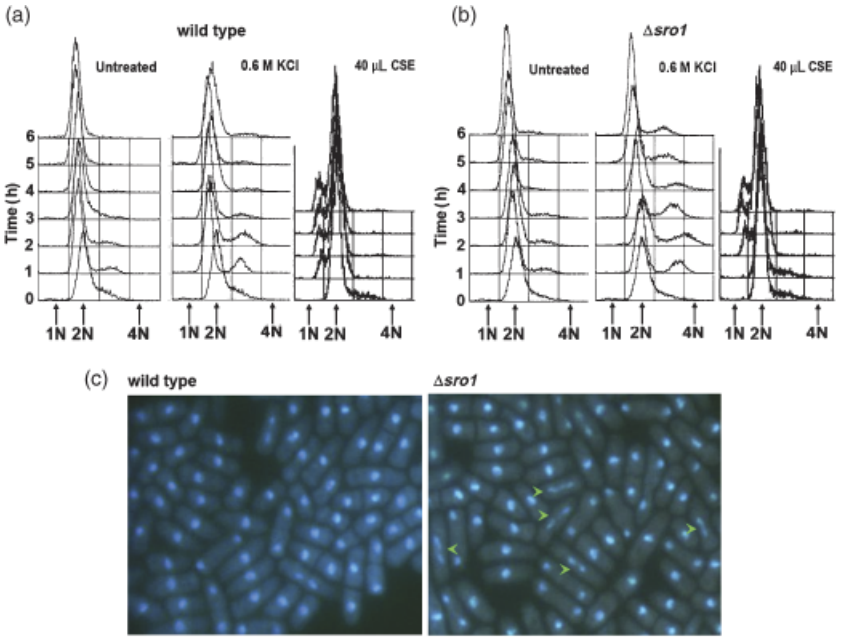

Fig. 6. *Sro1* can affect checkpoint activation during stress conditions. Asynchronously growing wild-type (a) and Δ*sro1* (b) cells were treated with 40 μL CSE or 0.6 M KCl, cells were collected and fixed at the indicated time points, processed ('Materials and methods') and then the DNA content was analysed on BD FACSCalibur. (c) Wild-type and Δ*sro1* cells were treated with 0.6 M KCl and stained with diamidino-2-phenylindol. 'Stretched Chromatin' type phenotype was observed in Δ*sro1* cells (indicated by arrowheads in the images).

nature of cell-cycle checkpoint activation in stressed *S. pombe* cells. DNA damage was also found to accumulate in Δ*sro1* cells treated with 0.6 M KCl, supporting the possibility of a role for sro1 in checkpoint activation after DNA damage. Here again, how *Sro1* functions is apparently determined by the nature of the stress stimulus. p38, the human homolog of *Sty1,* has been known to have major effects on mitochondrial membrane transition as well as free radical generation (Anoopkumar-Dukie *et al*., 2005; Kong *et al*., 2005). A similar role might be possible for the SAPK Sty1 in *S. pombe*. It is possible that *Sro1* could be a part of the mechanism for Sty1-dependent regulation of mitochondrial functions in *S. pombe*.

Thus, with respect to both ROS accumulation and cell-cycle progression, the function of sro1 is clearly stress-specific, so *Sro1* may play an important role in determining the specific differences in the responses of *S. pombe* to different stress stimuli. It is possible that a decrease in ROS levels with concomitant alteration in the temporal ROS generation profile and the differences in checkpoint activation are all indirect manifestations of the actual function of this novel protein. Presumably, Sro1 may be playing a role in an important basic cellular process that could affect so many other processes. Further studies are however required to confirm this possibility.

In all, this study reports the characterization of a novel stress-responsive protein in *S. pombe* and implicates it as an important player in *S. pombe* stress responses. Future studies based on the observations presented in this study may bring to light new facets of the *S. pombe* life cycle, especially with respect to the regulation of stress-specific cellular responses.

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