The oxidative and osmotic stress responses of *S. cerevisiae*

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First, wise words from a Nobel laureate, and no, not in science

> ”Everything is vague to a degree you do not realize until you have tried to make it precise.”

> ”The good life, as I conceive it, is a happy life. I do not mean that if you are good you will be happy - I mean that if you are happy you will be good.”

Bertrand Russell

And speaking of happiness

> ”Most folks are about as happy as they make up their minds to be.”

Abraham Lincoln

Till mamma och pappa och min bror

The oxidative and osmotic stress responses of *S. cerevisiae*

Abstract

The yeast Hog1 is a stress responsive mitogen activated protein kinase (MAPK) similar to mammalian p38 and JNK. Rck2 is a protein kinase downstream of Hog1. The Hog1 pathway was previously implicated exclusively in the response to osmotic stress.

This thesis investigates the role of the Hog1 MAPK signaling pathway in global post-transcriptional regulation and survival during environmental stress. We have shown that mutations in Hog1 pathway components make cells sensitive to oxidative and heavy metal stress and that Hog1 and Rck2 are activated during these stress conditions. Putative downstream components involved in oxidative stress resistance are identified.

Rck2 has a profound effect on the translational apparatus. During stress, Rck2 prevents polysome levels from falling too low. A kinase-dead allele of Rck2 confers stress sensitivity, and causes inactive polysomes to persist bound to mRNA during stress. Transcripts encoding translational components are deregulated in *rck2* mutants.

In response to osmotic stress, the Hog1 pathway affects mRNA levels of several hundred genes. This is accomplished to a large extent by regulation of mRNA stability, which is fast, widespread, and specific for several large groups of genes. Regulation of mRNA stability is dynamic throughout the response and precedes accumulation as well as decline of transcript levels. Unexpectedly, Rck2 has a larger effect on total transcript levels than on stability, indicating a role in transcription.

A comparison between two large scale studies reveals a positive global correlation between changes in polysomal association and mRNA stability in the adaptation phase. No global correlation was found in the early response. Among genes annotated as stress responsive, two groups were found which differed in their regulation at the polysomal level early in the response, and these were used to discover new genes with a characteristic post-transcriptional behavior after stress. Transcripts encoding components of the cytoplasmic translational apparatus were divided into three separate groups with a characteristic behavior. A group of genes shown to be translationally upregulated was shown to also be stabilized in response to stress.

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I – IV:

I. Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance.
Elizabeth Bilsland, Claes Molin, Swarna Swaminathan, Anna Ramne and Per Sunnerhagen,
Mol. Microbiol., 2004. 53(6):1743-56.

II. Rck2 is required for programming of the ribosomes during oxidative stress
Swarna Swaminathan, Tomas Masek, Claes Molin, Martin Pospisek and Per Sunnerhagen,
Mol. Biol. Cell., 2006. Mar;17(3):1472-82.

III. mRNA stability changes precede changes in steady – state mRNA amounts during hyperosmotic stress.
Claes Molin, Alexandra Jauhiainen, Jonas Warringer, Olle Nerman and Per Sunnerhagen,
RNA, 2009. Apr;15(4):600-14.

IV. A comparison between regulation of mRNA stability and polysomal association during osmotic stress.
Claes Molin, Janeli Sarv, Olle Nerman and Per Sunnerhagen
Manuscript.

Contents

Introduction

Living sketch

The little yeast *Saccharomyces cerevisiae* – you've all seen it in those small funny-smelling yellow packages in the store (at least if you've baked bread in Sweden) – is one of those organisms whose fate it has been to become a *model organism*. A sketch to help us understand the great mysterious masterworks of the (known) universe: living things. Yeast contains a lot of the basic mechanisms that are present in our cells too. It kind of lacks the added dimensions of cellular specialization and cell-to-cell communication (although varieties of these exist, such as pseudohyphal growth, meiosis and pheromone signaling), but the groundwork is there: DNA, the tightlipped spiral – the blueprint of life tattooed on its form, and RNA and proteins, the workhorses who just won't stop surprising.

I honestly never thought doing this would be so difficult. I know this says more about me than about yeast. But I just wanted to know how an assembly of atoms comes alive. How difficult could that be?

Pretty difficult, yes. Is it even understandable? But maybe I asked the wrong question to begin with: maybe what I fuzzily asked for was: what is the *idea* of life? What I'm seeing is the prerequisite for it. The wiring. Life is not just complex – it is complexity. Without complexity (i.e. myriads of components in networks so vast you just want to lie down) – no life. Zillions of interactions makes life, just like zillions of human interactions makes a civilization, something more than the sum of its parts. Puzzling out all these interactions will surely be done someday. A computer will keep track of them. Maybe yeast will be the first? Will we then know life?

Anyway. I looked at a small part of the wiring. Yeast is not human, that's for sure. But it's a sketch. And it *is* alive.

The yeast *S. cerevisiae*

Physiology, life cycle and genome

Saccharomyces cerevisiae (*S. cerevisiae*) is a eukaryotic, unicellular and non-motile yeast belonging to the spore-forming phylum *Ascomycota*. *S. cerevisiae* occurs naturally in soil and water, but has for thousands of years been used by man in fermentative processes such as bread baking and alcohol brewing. Hence the

name cerevisiae, which is Latin for ”of beer”. Also, ”Baker’s yeast” is one of several common names.

S. cerevisiae is preferentially diploid and multiplies asexually by budding (which earns it another common name: ”budding yeast”). During exponential growth in rich medium in the laboratory *S. cerevisiae* has a generation time of about one and a half hours. Upon reaching a certain density in the medium, the cells enter a stationary phase during which metabolic activity drops and the cells become quiescent. In certain conditions such as nitrogen starvation, *S. cerevisiae* cells may arrest in G1, undergo meiosis and sporulate. The spores can withstand harsh conditions until they are resuscitated by the addition of nutrients, whereupon the haploid cells quickly mate to become diploid again. *S. cerevisiae* has two mating types, MAT**a** and MAT**α**, and can normally switch between the two. In laboratory strains, however, this ability is usually abolished to prevent undesired exchange of genetic material.

In 1996, the *S. cerevisiae* genome was the first eukaryotic genome to be completely sequenced (24). The genome contains some 6603 ORFs (1) spanning 12,8 Mb on 16 chromosomes. Around a hundred million years ago, the *S. cerevisiae* genome underwent a duplication event (69). This was followed by partial loss of duplicated segments, which means that a large fraction of all genes exist as two closely related copies. The *S. cerevisiae* genome is dense compared to higher eukaryotes, with about 70 % coding sequence.

Environmental stress

Since yeast is a free-living single celled organism, the external milieu can vary greatly. If the variations are sudden or extreme, the cell may come under stress. Yeast is equipped with several elaborate defense mechanisms against different types of stress imposed by the environment, and many of these are similar to mechanisms in higher organisms. This thesis concerns stress caused by reactive oxygen metabolites, heavy metals and changes in external osmolarity and salt concentration.

Oxidative stress

A side effect of respiration is the production of reactive oxygen species (ROS) that can damage proteins, lipids and DNA (26). The origin of most ROS is the superoxide anion, which is a side product from the activities of NADH dehydrogenases Nde1 and Nde2 and cytochrome *bc1* in the mitochondria. The superoxide anion is detoxified in two steps, first into hydrogen peroxide by

superoxide dismutases, and then it is fully reduced into molecular oxygen and water by different peroxidases. In certain conditions however, hydrogen peroxide may be partially reduced and generate the hydroxyl radical (OH·), which is extremely reactive and damaging.

There are two main types of peroxidases; the glutathione peroxidases (GPX), which use reduced glutathione as electron donors, and peroxiredoxins, which use thioredoxin. Both glutathione and thioredoxins are also important in regulating the redox state of sulphur-containing proteins, and the biosynthesis of sulphur amino acids, Fe/S clusters and dNTPs. Glutathione, a tripeptide with a cysteine acting as the proton donor, is an important reducing agent in the cell. Enzymes involved in glutathione metabolism are consequently upregulated during oxidative stress (Paper II). The main route for this upregulation is the transcription factor Yap1, which directly senses oxidative stress (39).

The toxic and carcinogenic metal ion cadmium (Cd^{2+}) has been shown to cause oxidative damage in lungs, liver and kidney in animals. Cd^{2+} induces ROS production in rat liver tissue (34), possibly by depleting the cellular supply of glutathione.

An interesting twist to the story of ROS, for long considered nothing but evil to the cell, has emerged in recent years as it is becoming clearer that ROS have a role in intracellular signaling. This is exemplified in the fruit fly immune system, where ROS influences the differentiation of blood cell progenitors (40).

Osmotic stress

Increased osmolarity in the environment causes water to flow out of the cell by osmosis. Counteracting osmotic changes is important for any cell, free-living or organism-bound, and this means that a fast response to osmotic stress is necessary. Osmotic stress triggers an acute response aimed at recreating the osmotic balance between the intracellular and extracellular spaces. Yeast cells accomplish this by several mechanisms, of which the most important in the acute phase is the production of glycerol as an osmolyte (28). Also, as will be discussed in more detail below, the acute phase includes regulation of many aspects of cell life such as the cell cycle, plasma membrane transport, nutrient metabolism, and transcription and translation of genes and gene products. In the laboratory, sodium chloride or sorbitol is often used to induce osmotic stress. In the papers of this thesis sodium chloride is used and it should be noted that this causes sodium toxicity an addition to osmotic stress. After mild sodium chloride shock (0.4 M), yeast stops growing in rich liquid

medium for about five hours, after which exponential growth is resumed. This means that the study of this response involves looking at a transient phenomenon. True to this notion, large scale transcriptional analysis early on revealed a transient upregulation of mRNAs in response to osmotic stress followed by a return to normal levels (discussed below). The response at the transcriptional level includes both genes specific for this type of stress and genes that are also involved in several other stresses, forming a group of genes called the ”environmental stress response”. It is an interesting fact that not many of these upregulated genes are also detected in mutational analyses as being sensitive to stress, although important exceptions certainly exist.

The Hog1 MAP kinase pathway

Architecture of the Hog1 MAP kinase pathway

MAPK (mitogen-activated protein kinase) pathways are signaling pathways involved in several different physiological processes, and conserved in eukaryotes. The core module of MAPK pathways is a triad of proteins through which a phosphorylation signal is sequentially transmitted. These proteins are called MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs) and MAPKs. The central and defining of these is the MAPK. *S. cerevisiae* possesses five different MAPKs, involved in the protection against environmental stress (Hog1), mating (Fus3), filamentous or pseudohyphal growth (Kss1), cell wall integrity (Slt2) and spore formation (Smk1),

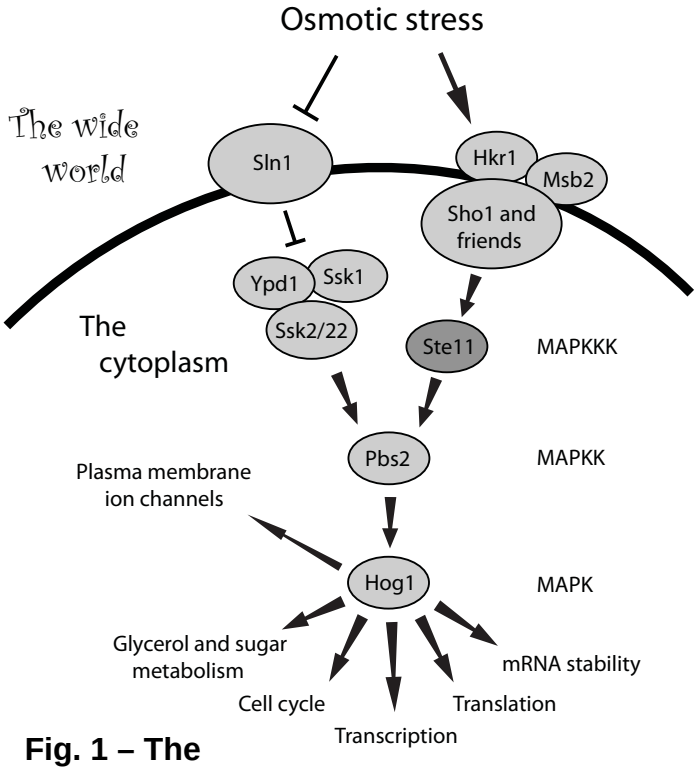

Fig. 1 – The Hog1 pathway

respectively.

One of the most important functions of MAPK pathways is to signal the detection of cellular stress of different kinds. In mammals, the stress regulated p38 and JNK MAPK pathways control important cellular responses such as inflammation and apoptosis. The Hog1 MAPK pathway (Fig. 1) has mostly been implicated in the response to osmotic stress (9), but it is also activated during oxidative stress, as shown in Paper I of this thesis, heat stress (68), citric acid stress (33) and cold shock (41). Even though each MAPK pathway includes the core triad of kinases, the architecture of the different pathways is indeed much more complex. The Hog1 MAPK pathway has only one MAPKK, Pbs2, but is has three different MAPKKKs: Ste11 and the redundant Ssk2 and Ssk22. Two separate upstream osmotic stress sensing branches, called the Sho1 and the Sln1 branches, control Ste11 and Ssk2/Ssk22 respectively. Sln1 is a plasma membrane protein that senses membrane turgor, and is actually a constitutively active negative regulator of Hog1 during normal osmotic balance. During osmotic stress, Sln1 is inactivated and the Ssk2/Ssk22 MAPKKKs are activated through a phosphorelay system including Ypd1 and Ssk1. In the case of the Sho1 branch it has for long not been clear what constitutes the actual osmosensor, but recent work suggest it may be the mucin-like transmembrane proteins Hkr1 and Msb2 (59). The plasma membrane Sho1 transmits the signal to the MAPKKK Ssk1 together with Opy2, another transmembrane protein, the adapter protein Ste50, the PAK family kinase Ste20 and the GTPase Cdc42.

Components of both upstream branches of Hog1 have been shown to be involved in oxidative stress resistance (56). We have shown that Hog1 is activated by oxidative and heavy metal stress (Paper I). Although Pbs2 is important for Hog1 phosphorylation, it is not known from where the signal originates. There are several examples in mammals, *Drosophila*, and the fission yeast *S. pombe*, of oxidative stress influencing MAPKKK activity, although similar mechanisms are yet to be found in budding yeast. For example, the human MAPKKK ASK1 is activated through dimerization and autophosphorylation following dissociation of oxidized thioredoxin from ASK1 during oxidative stress (36). Also, another MAPKKK, MEKK1, is inhibited during oxidative stress by glutathione-induced reduction of a cysteine residue in the ATP-binding site (12).

Following oxidative stress, *S. pombe* MAPK Sty1 is activated via a two component system similar to the Sln1 branch (48). The signal is mediated to the

transcription factor Atf1, which Sty1 controls both by phosphorylation and mRNA stability regulation through the mRNA binding protein Csx1 (51).

The role of Hog1 in regulation of transcription, cell cycle progression and ion transporter activity during osmotic stress

The activation of the Hog1 pathway during osmotic stress is very well studied and has many effects. First, Hog1 regulates transcription. Upon activation by osmotic stress, phosphorylated Hog1 within minutes accumulates in the nucleus. This leads to the transcription of between 50 and 150 genes involved in different cellular processes such as carbohydrate metabolism, ion transport and defense against oxidative stress (11, 22, 43, 50, 70). Of these, genes involved in glycerol production are especially important (2). Several transcription factors such as Msn2, Msn4, Sko1, Smp1 and Hot1 take part in this process and additionally, Hog1 has been reported to interact with RNA polymerase II, histone deacetylases, and directly with promoters indicating a complex involvement in transcriptional regulation. Also, in Paper III, we show that the Hog1-activated kinase Rck2 affects transcription. A large group of genes is also transcriptionally repressed during osmotic stress. These predominantly consist of genes involved in protein synthesis. An indirect effect of Hog1 on transcription is the repression of genes involved in the Kss1 MAPK pathway through the phosphorylation of the Kss1 target Tec1, preventing cross-talk between the two MAPK pathways (55).

Second, according to a recent study, despite the extensive transcriptional regulation that Hog1 instigates, the acute life saving effect of Hog1 during osmotic stress takes place in the cytoplasm and involves the production of glycerol (67). This effect involves inhibition of the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, which redirects glycolysis to production of dihydroxyacetone phosphate (DHAP), a precursor of glycerol. Furthermore, stimulation of Hog1 leads to activation of 6-phosphofructo-2-kinase. This enzyme catalyses the production of fructose 2,6-bisphosphate, a signaling molecule that induces increased flux through glycolysis, allowing more sugar for DHAP production.

Third, Hog1 has been shown to regulate the activity two plasma membrane ion channels, Nha1 and Tok1, in response to 0.4 M of sodium chloride (45).

Fourth, Hog1 regulates the cell cycle by activating two checkpoints: the G1/S checkpoint and the G2/M morphogenesis and septin checkpoint, probably depending on where in the cell cycle the cell finds itself during stress. The G1/S checkpoint is activated both through phosphorylation of the CDK-inhibitor Sic1 and

downregulation of the G1 cyclins Cln1 and Cln2 (17). In G2/M, the checkpoint is activated through phosphorylation of Hsl7, which allows the CDK-Cyclin B inhibitor Swe1 to accumulate.

Fifth, Hog1 regulates translation through the phosphorylation of Rck2. Rck2 phosphorylates the eukaryotic elongation factor-2 (eEF-2, or Eft2) in response to salt stress, which is thought to inhibit translational elongation (38, 60). Also, Hog1 is involved in regulation of polysome association during severe salt stress (37). In Paper II we show that Rck2 is involved in the regulation of ribosome association to mRNA during oxidative stress.

And finally, it has earlier been shown that Hog1 regulates stability of yeast and mammal transcripts through the RNA binding protein Pub1 (65). We have shown that Hog1 regulates mRNA stability of almost all of the transcriptionally upregulated genes in a coordinated manner in response to osmotic stress (Paper III). This is in accordance with a recent paper where Hog1 was found to influence stability of stress-induced transcripts (52).

Following adaptation to osmotic stress, Hog1 is downregulated by cytoplasmic and nuclear phosphatases. This is an important mechanism since overactive Hog1 is detrimental to the cell.

The response to osmotic stress is more exclusively mediated by Hog1 during mild than severe stress (37, 50, 62). Furthermore, the response to severe osmotic stress is delayed as compared to mild stress, which is reflected in a delayed nuclear accumulation of Hog1 as well as delayed upregulation and translational regulation of stress responsive genes. This could be a result either of intracellular dysfunction in the early phase or of other responses taking priority.

As mentioned previously, activated Hog1 suppresses other MAPK pathways. Prevention of crosstalk between signaling pathways is an interesting subject and has been the object of many studies. In a *hog1Δ* deletion mutant, osmotic stress induces transcription of genes involved in mating and pseudohyphal growth, which underlines the importance of crosstalk regulation of MAPK pathways.

Rck2

The yeast MAPK activated protein kinases (MAPKAPKs) Rck1 and Rck2 were discovered in 1994 (14). They were initially described as suppressors of cell cycle checkpoint mutations in *S. pombe* (13), and in about the same time Rck2 was found to be structurally similar to type 2 Ca^{2+}/calmodulin dependent protein kinases (38).

Several roles have been ascribed to Rck2, while the function of Rck1 has been more elusive. Around 2000, two groups independently performed two-hybrid screens that revealed Rck2 to be a substrate of Hog1 (7, 60). Rck2 was also shown to be phosphorylated by Hog1 during osmotic stress (7). Although an *rck2Δ* mutant is not very sensitive to osmotic stress, overexpression rescues a *hog1Δ* mutant during osmostress. However, overexpressing an allele of Rck2 that is incapable of autophosphorylation (a ”kinase-dead” allele, *rck2-kd*) makes the cell sensitive to stress. Furthermore, deletion of *RCK2* rescues cells with an overactive Hog1 pathway, which otherwise is detrimental for reasons not fully understood. It has also been shown that Rck2 is involved in translational regulation through stress-induced phosphorylation of Eft2 (60).

More evidence for a role in translational regulation comes from Paper II, where Rck2 is shown to positively influence the association of the mRNA pool to ribosomes during oxidative stress. Furthermore, the kinase-dead *rck2* mutant shows deregulation of polysomal association that makes the cell highly sensitive to oxidative stress.

Given the many clues to a function in post-transcriptional regulation of Rck2, it was surprising to find that *rck2Δ* mutants seem more defective in transcription than in regulation of mRNA stability during osmotic stress (Paper III). In fact, *rck2Δ* cells were almost as defective in regulating steady state level mRNA in response to osmotic stress as were *hog1Δ* cells. In contrast, during oxidative stress Rck2 did not appear to influence transcription, although steady state levels of cytoplasmic ribosomal protein (cRP) transcripts were higher in unstressed *rck2Δ* than in wild type cells (Paper II).

Mammals contain several classes of MAPK activated kinases, of which three belong to the ”real” MAPKAPK subfamily (19). These are not clear-cut structural homologs of Rck1 and Rck2 except for in the actual catalytic domain where structural similarity is high. The mammalian MAPKAPKs are activated by the stress-regulated MAPK p38 and have many different functions such as regulation of mRNA stability, cell cycle checkpoint signaling and chromatin remodeling, which possibly influences deregulation of stress-responsive gene transcription.

Like *S. cerevisiae*, *S. pombe* contains two MAPKAPKs: Mkp1 and Mkp2, also known as Srk1 and Cmk2, respectively (3, 6, 54, 58), these kinases have been implicated in cell cycle regulation, meiosis, oxidative stress resistance and translation.

Post-transcriptional regulation

Regulation of translation

The central dogma of molecular biology was postulated by Francis Crick in the fifties and holds that proteins - the work horses of the cell - are produced from an mRNA template which is in turn produced from a DNA template. Each of these steps is carefully regulated by mechanisms that include both general and unique components for different genes. Translation, the production of a polypeptide chain from an mRNA, requires the mRNA to be modified by a cap structure in the 5' end and polyadenylation in the 3' end. Moreover, several proteins need to be bound to the mRNA, such as initiation factors and poly-A binding proteins, creating what is called a messenger ribonucleoprotein (mRNP). The actual polypeptide elongation is carried out by the ribosome, a large protein-RNA complex composed of one large and one small subunit. The small (40S) subunit is the first to bind the mRNA as a complex called the pre-initiation complex, which also includes a charged initiator tRNA and the initiation factor eIF2 (Fig 2a). The pre-initiation complex docks to initiation factors (eIFs) at the 5' end of the mRNA and then scans the mRNA for a translation start codon (Fig. 2b). When a start codon (AUG) is found, the large (60S) subunit joins the small subunit and translation is initiated (Fig. 2c). Translational elongation is controlled by four elongation factors, Eft1, Eft2 and Hyp2 and its paralog Anb1 (Fig. 2d). Eft2 can be regulated by phosphorylation by Rck2 as noted

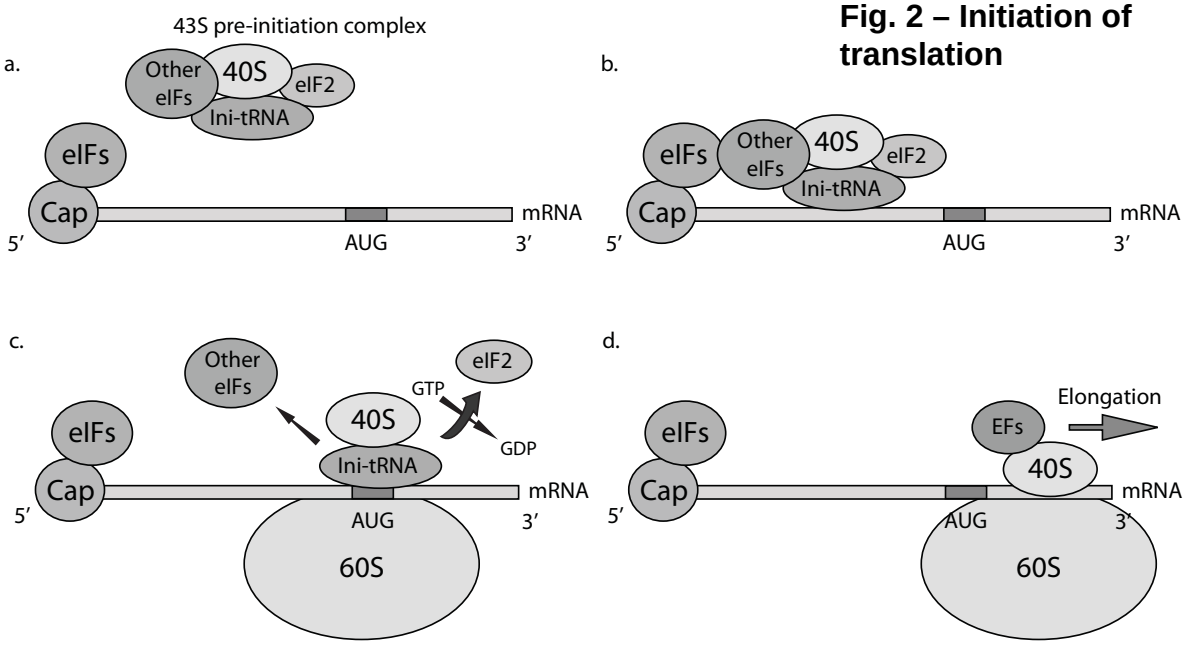

Fig. 2 – Initiation of translation

previously. In fission yeast, the MAPK Sty1 has been shown to interact with elongation factor 2 (5).

During normal growth as well as in several stress conditions, translation is specifically regulated for certain transcripts (4, 32, 37, 44, 57). Translation may basically be regulated at either the initiation step or the elongation step, but since initiation is a more complicated process, it seems reasonable that more control points are also present at this step. Translation initiation is inhibited in a range of conditions such as amino acid and glucose starvation, oxidative stress and membrane dysfunction (15). One important control point is the above mentioned eIF2. eIF2 is a protein complex that includes Sui2 (eIF2α), which in its GTP-bound form can interact with initiator-tRNA and initiate translation. Phosphorylation of Sui2 induces it to inhibit its own GTP/GDP exchange factor, which leads to an inhibition of translation initiation. This phosphorylation occurs at a conserved residue in response to several different kinds of cellular stress including sodium chloride, oxidative stress, and amino acid deprivation (which is the most studied, and the mechanism and its consequences are really elegant – but, alas, not within the scope of this text) (27, 35). Another mechanism through which initiation is controlled is by inhibiting binding of the eIF4E complex to the 5' cap. This is accomplished by competitive binding of eIF4E by eIF4E-binding proteins, which are activated by oxidative stress (35) and membrane dysfunction.

Regulation of mRNA stability

mRNA transcript levels are the sum of production and degradation. mRNA degradation is a highly regulated process which has been shown to play a crucial part in the modulation of mRNA levels both during regular proliferation and in many stress conditions (18, 20, 25, 46, 53, 63).

mRNAs can be degraded from 5' to 3' by the exonuclease Xrn1 and from 3' to 5' by the exosome complex (21). In yeast, the two mechanisms appear partly redundant since knocking out one of them has little effect on mRNA levels. Since mRNAs are protected at the 5' end by the cap structure, and at the 3' end by the poly(A)-tail, these structures usually need to be removed prior to degradation, although exceptions exist. Regulation of mRNA stability can be mediated both by targeting the general degradation machineries and by specifically targeting certain mRNAs. Several RNA binding proteins have been shown to influence stability for example by recruiting components of the degradation machinery to target transcripts. In yeast, the RNA binding Pub1 targets at least two different kinds of mRNA sequence

elements and regulates the stability of several hundred transcripts (16). Other yeast examples include Cth1 and Cth2, which regulate transcripts involved in iron and carbohydrate metabolism during iron deficiency (47), and the Puf proteins 1 through 6, which bind large and distinct classes of mRNAs during normal growth (23), and some of which have also been implicated in translational regulation. Among the many other varieties, there is also the interesting case of the ribosomal protein encoding Rps28B that binds to and negatively regulates the stability of its own mRNA (21).

In yeast and other organisms, MAPK pathways have been shown to influence mRNA stability through RNA binding proteins, which often bind to regulatory elements in the 3'-UTRs of target transcripts. The mammalian Hog1 homolog p38 affects several RNA binding proteins, such as KSRP, TTP and HuR, affecting the stability of their respective mRNA targets (21). In *S. pombe*, the stress-induced MAPK Sty1, in conjunction with another kinase, regulates the stability of the Atf1 transcription factor mRNA in response to oxidative stress through the RNA binding protein Csx1 (30, 51). Finally, Hog1 itself affects the stability of the translational elongation factor Hyp2 (Tif51A) through Pub1 in a glucose dependent manner (66).

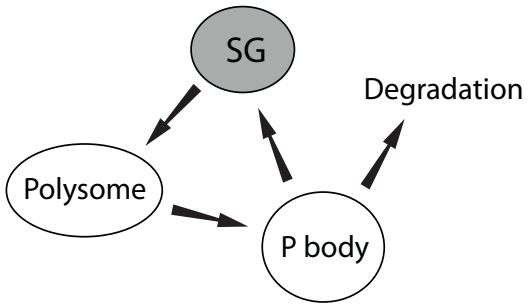

Fig. 3 – mRNPs

Regulation of mRNA stability is tightly coupled to translational regulation. Inhibition of translational elongation tends to stabilize transcripts on polysomes (mRNAs with more than one ribosome bound) while inhibition of initiation sends them to dynamic ribonucleoprotein complexes called processing bodies (P bodies) (8, 10). P bodies contain mRNA decay factors such as the exonuclease Xrn1 and the decapping enzyme Dcp2, but also parts of the translational machinery, and have been proposed to function in mRNA degradation, storage and sorting. For example, in yeast, mRNAs appear to be channeled through P bodies after glucose deprivation to distinct mRNP complexes called stress granules (SG), which are formed when translation initiation is rate limiting. SGs contain translation initiation factors, but also Pub1, which may stabilize transcripts during stress. To sum up, cells contain several different mRNPs such as polysomes, P bodies and SGs, and the cycling between these affects translation and mRNA decay. These mRNPs should however be viewed as a dynamic continuum rather than static and sharply defined structures.

Moreover, research in this area seems to be just getting started, and many more aspects of mRNA metabolism is likely to be uncovered in the coming years.

Present study

Rck1 and Rck2 are required for oxidative stress resistance (Paper I)

Results

pbs2Δ and *rck2Δ* mutants are sensitive to oxidative stress induced by tert-butyl hydrogen peroxide (tBOOH). *rck1Δ* displays moderate sensitivity. *pbs2Δ* and *rck2Δ* also display sensitivity to cadmium stress. A *hog1Δ rck2Δ* double mutant is not more sensitive than either of the single mutants. Overexpression of *RCK2* partially complements the sensitivity of *pbs2Δ* mutants.

Hog1 is phosphorylated in a Pbs2-dependent manner after stress induced by tBOOH, H_2O_2 and Cd^{2+}. The intensity of the phosphorylation is comparable to that induced by osmotic stress, but the kinetics is much slower – the peak of phosphorylation is at 60 min., compared to as soon as after 5 min. after osmotic stress. Rck2 is also phosphorylated after tBOOH stress but to a substantially lesser degree that during osmotic stress. This is partially dependent on Pbs2.

Hog1 is partially translocated to the nucleus after tBOOH stress. In an *rck2Δ* mutant, this is augmented. Even in unstressed cells, Hog1 is partially nuclear in an *rck2Δ* mutant.

Studies of physical interactions revealed that Rck2 and Rck1 bind to substrates involved in oxidative stress resistance. Two proteins, Yap2 and Zrc1, were further tested and shown to be genetically downstream of Rck1 and Rck2, respectively.

Discussion

Prior to this paper, only osmotic stress had been shown to induce Hog1 phosphorylation. Here we show that oxidative stress induces Hog1 phosphorylation and partial nuclear translocation, albeit with slower kinetics than during osmotic stress. In most organisms MAPK pathways respond to a range of stresses, and several stress conditions have now been shown to activate the Hog1 MAPK pathway in *S. cerevisiae* (33, 41, 68). Rck2 is phosphorylated in a partly Pbs2-dependent way with the same kinetics as Hog1, and this plus the fact that *hog1Δ* and *rck2Δ* mutations are epistatic and that Rck2 overexpression complements a *pbs2Δ* mutant places Hog1 upstream of Rck2 during oxidative stress. Still, the fact that Rck2 is partially phosphorylated in cells lacking *PBS2* indicates that more pathways than Hog1 phosphorylate Rck2 during oxidative stress.

The diverse effects of different ROS-generating compounds have been noted elsewhere. Mascarenhas *et al*. have shown that oxidative stress inhibits translation at initiation as well as elongation, but that different compounds to different degrees rely on eIF2 for inhibition of initiation (35). That is, inhibition of initiation by hydrogen peroxide relies completely on eIF2, while in the case of cadmium and diamide, regulation of eIF4E binding is also involved. Since Rck2 is involved in the translational response to oxidative stress, this might be an answer to why different oxidative stress inducing compounds affect Hog1 pathway mutants differently. It is however important to note that Hog1 and Rck2 are not phosphorylated until after 30 min. of stress, while the effects of polysomal association is seen already after 15 min. (with a difference between wt and *rck2Δ*, Paper III). This could either mean that phosphorylated Hog1/Rck2 is not important in the early phase, or that the conditions used in the experiments were different enough to cause differences in timing of the response. Rich medium and 3 mM tBOOH were used in Paper I, while synthetic medium and 0,8 mM of tBOOH were used in Paper II. It is known that more severe stress causes a delay in the response during hyperosmotic stress, which could mean that the polysomal effects would occur later during the conditions used in Paper I. Alternatively, phosphorylated Hog1/Rck2 is not important in this phase, but at a later time. However, since polysomal association is not recuperated even after mild oxidative stress after 120 min. (Paper II, Fig. 2), it seems that phosphorylated Hog1/Rck2 is not involved in polysomal recruitment during recuperation.

Rck2 affects the nuclear accumulation of Hog1 in response to oxidative stress, which might be because cells lacking Rck2 are more stress sensitive. Possibly, Rck2 functions as a cytoplasmic anchor for Hog1. The interaction between Rck2 and Hog1 is robust, supporting this idea (7).

Rck2 is required for programming of ribosomes during oxidative stress (Paper II)

Results

Overexpression of an inactive *RCK2* allele (*rck2-kd*, ”kinase dead”) leads to sensitivity to oxidative stress caused by tert-butyl hydrogen peroxide. Even unstressed cells overexpressing Rck2-kd have decreased viability.

After oxidative as well as osmotic stress, wild type cells display a decrease in translation as measured by a fall in levels of mRNA bound to polysomes. In *rck2Δ* mutants, polysome levels fall even more, while in cells overexpressing Rck2-kd, the drop in polysome levels is much delayed. However, the polysomes present in *rck2-kd* cells after stress seem to be inactive, as judged by measurements of amino acid incorporation into proteins.

In response to oxidative stress, genes involved in sulphur metabolism such as the biosynthesis of sulphur-containing amino acids and glutathione are upregulated and the corresponding transcripts also associate more to polysomes. This effect is not dependent on Rck2.

Levels of cRP go down after oxidative stress. The levels of this kind of proteins are raised in the unstressed condition in both *rck2Δ* and *rck2-kd* mutants.

NB: Since the microarray data set contained a flaw in the form of saturated spots, these data were later reworked. It was then assumed that spots in the medium range would best reflect true values, and the reworking entailed down-weighing of (*i.e.* giving less influence to) intense spots on filters with high mean intensity as well as down-weighing of weak spots on filters with weak mean intensity. As a consequence of this reworking, the conclusions from Fig. 7 of this manuscript that abundant mRNAs associate differently with polysomes in *rck2Δ* and *rck2-kd* cells is no longer believed valid. However, the other points of the manuscript still hold.

Discussion

Following oxidative stress, global translation is inhibited due to depression of polysomes, which is reflected in a drop in protein synthesis. This is similar to the situation during osmotic stress (61). However, with regard to osmotic stress there are conflicting data regarding the involvement of the Hog1 MAPK pathway in protein synthesis regulation, especially during the early phase (60, 61). Uesono *et al*. looked at longer time points and claim that Hog1 is needed during the late adaption phase

but not for immediate downregulation of protein synthesis, while Teige *et al*. claims that Hog1 is needed for early inhibition of translation. Our finding that Rck2 prevents an exaggerated drop in polysome levels early during oxidative stress indicates that Rck2 is important for active translation, and hence could play a role in adaptation or recovery. Surprisingly, an inactive allele of Rck2, Rck2-kd (incapable of autophosphorylation (49)), displayed the opposite phenotype: these mutants were unable to depress polysomes at the wild type rate. This might mean that Rck2 incapable of autophosphorylation freezes polysomes on mRNA in an inactive state. Strains overexpressing Rck2-kd are more sensitive to stress, which might mean that freezed polysomes are detrimental.

The transcriptional and translational responses to oxidative stress essentially potentiate each other, meaning that transcripts that are transcriptionally induced also get translated more. Rck2 however, seems not to be involved either of these responses, unlike the case in osmotic stress (Paper III and (64)).

Since the levels of cRP transcripts are raised even in unstressed cells when *RCK2* is deleted, regulation of cRP gene transcription may be influenced by Rck2. However, the polysomal levels of these transcripts are not increased in the unstressed condition. Rck2 was not involved in transcriptional regulation during oxidative stress, as it is during osmotic stress (Paper III).

Changes in mRNA stability precedes changes in mRNA steady-state levels during osmotic stress (Paper III)

Results

Following mild hyperosmotic stress (0.4 M NaCl), several categories of genes well known to be transcriptionally upregulated are also regulated at the level of mRNA stability. The amplitudes of the mRNA stability changes are predicted to influence the steady state levels to a substantial degree. Early in the stress response (after 6 min.), several categories of genes involved in the stress response increase in mRNA stability, while later in the response (30 min.), these gene categories are clearly destabilized. Other gene categories, such as those involved in ribosome biogenesis and assembly and RNA metabolism, are stabilized late in the response.

Hog1 influences the stability of transcriptionally induced as well as repressed genes. For induced genes, early stabilization as well as late destabilization is

affected. For repressed genes, the initial weak destabilization in the wild type is exaggerated in a *hog1Δ* mutant, while the late stabilization is less pronounced.

Different gene categories behave differently with respect to changes in mRNA stability, and the changes in stability are reflected in the steady-state levels during the later stage of the response. Transcripts involved in amino acid metabolism are stabilized throughout the response, while they also increase in steady state levels. Transcripts involved in phosphate and carbohydrate metabolism are stabilized early and destabilized later in the response, as are genes involved in the response to stress. Transcripts involved in RNA modification, ribosome biogenesis and mRNAs encoding ribosomal proteins (RP) are destabilized late in the response. Early in the response however, transcripts involved in RNA modification are destabilized while transcripts belonging to the other two groups are stabilized.

Genes belonging to the GO Slim category ”response to stress” contain two groups of transcripts whose stability is regulated. One group is stabilized early on while the other is not. The former group is also more strongly upregulated on the steady-state level, and contains many well-known osmotic stress-regulated transcripts such as *HOR2*, *TPS2* and *HSP42*. Hog1 influences both total levels and stability for both groups while Rck2 mainly influence total levels.

Transcripts encoding RP can be divided into three groups regarding regulation of mRNA stability: two containing mostly cRP, of which one is more stabilized than the other, and one group containing mostly mitochondrial RPs. Hog1 and Rck2 both affect stabilization of the former groups, as well as the recovery after one hour at the steady state level.

Discussion

The main conclusion of this manuscript is that almost all genes that show a change in total levels after osmotic stress also display a MAPK-mediated change in mRNA stability preceding the total level change. Especially with regard to upregulated genes, the mRNA stabilization is dependent on Hog1.

The apparent synchronization of the fluctuations in transcript stability and total levels, with different kinetics for different functional groups of mRNAs, suggests an elegant pattern of transcriptional induction and stability regulation which Hog1 controls. A likely possibility is that co-regulated mRNAs share sequence motifs which enables them to bind proteins that regulate their stability, and searching for such motifs within these functional groups might prove fruitful. Another possibility is of course to alter the sequence of some candidate transcripts and see if this affects

their stability regulation. Most stability-regulating sequences reside in the 3'-UTR, and this is an appropriate place to start looking.

Through a different experimental approach, Romero-Santacreu *et al*. (52) recently showed similar results as in this paper, together with direct measurements of transcriptional activity. However, Romero-Santacreu *et al*. could also reveal a global destabilization of all transcripts in the wild type, along with a global stabilization in a *hog1Δ* mutant, which we missed due to our setup. These results show that MAP kinases are able to induce fast, widespread changes in mRNA stability of the same target genes that are transcriptionally induced in response to stimuli.

Another recent paper by Kitchen *et al*. showed that activation of the Fus3 MAPK pathway involved in mating does not result in changes in stability of its target transcripts (31). This means that Hog1 and Fus3 have different effect on mRNA stability, or possibly that changes in mRNA stability is connected to transcriptional activation since the reporter transcripts used in Kitchen *et al*. was not transcribed from their endogenous promoters.

The use of 1-10 phenanthroline (Phen) was primarily based on the results from Grigull *et al*. (25), which compared different ways of measuring mRNA decay by transcriptional shutoff. This study compared the use of heat labile RNA polymerase II (in the *rpb1-1* mutant) with Phen (a metal chelator), 6-azauracil and cordycepin (nucleobase analogs), thiolutin (interacts with and inhibits RNA polymerases) and ethidium bromide (a DNA intercalator). In our paper, we wished to avoid the use of heat shock which is required for the use of the *rpb1-1* mutant, so we focused on Phen, which together with thiolutin displayed the best correlation with the decay pattern of *rpb1-1* mutants. In order to avoid looking at Phen-specific effects, we subtracted a list of genes shown to be specifically affected by Phen in the meticulous study of Grigull *et al*.

A recent paper by Pelechano and Perez-Ortin that investigates the effect of thiolutin on mRNA stability should be noted in this context (42). Here it was discovered that high doses of thiolutin inhibits mRNA decay in addition to transcription. This of course has a large impact on the measurements of mRNA half-lives, and since Phen showed the greatest correlation with thiolutin in Grigull *et al*., this question must be addressed. It should be expected that Phen, being a divalent ion chelator, has various impacts on cell metabolism in addition to transcriptional inhibition, which may very well extend to effects on mRNA decay. It is even so that one might expect effects on mRNA decay regardless of the method of global transcriptional shutoff, especially during later time points, since this must be a huge

stress for the cell. In paper III, the strategy employed was not so much to primarily minimize these side effects but to subtract them by comparisons. This should mean that unless Phen affects the wild type and the deletion mutants differently, or that it affects stressed and unstressed cells differently, the results should hold. Still, an experiment that could be performed is, like Pelechano and Perez-Ortin did, to titrate Phen against stressed and unstressed strains for some candidate genes. Reassuringly, the conclusions of Romero-Santacreu *et al*. agree well with our paper, although raw data has not been compared. (This could be an interesting project in itself.)

A comparison between regulation of mRNA stability and polysomal association during osmotic stress (Paper IV)

Results

Two experiments with identical setups differ with regard to timing of the response. The peak of the response in total mRNA levels is earlier in Warringer *et al*. (64) than in Molin *et al*. (Paper III).

Globally, polysomal association and mRNA stability did not correlate after 6 min. of stress. After 30 min., there was a positive global correlation. Looking at a group of previously known stress-induced genes, there was a negative correlation early and a positive correlation later.

The GO Slim category ”response to stress” contains two groups of genes that are regulated in both experiments and differ in that one is translationally upregulated early and the other is not. The former included transcripts involved in trehalose metabolism. These two groups were used as templates in searches for more genes regulated at the post-transcriptional level. Genes that are upregulated at the polysomal level but not at the total level early are stabilized during the late phase of the response.

cRP transcripts divided into three groups with respect to translational and mRNA stability regulation: one which is both translated and stabilized and two which are either stabilized or translated, respectively.

Discussion

The lack of global correlation at early time points seems in part to be due to biological and sampling variation since the response is delayed at the total level in Molin *et al*. This highlights the importance of rigorous protocols when studies are to

be compared directly. Still, some groups are regulated in both experiments, providing an opportunity for examining co-regulation at the post-transcriptional level. The fact that cytoplasmic protein transcripts fall into three different groups with regard to the relation between translation and mRNA stability is interesting. This might indicate different targeting to mRNPs such as polysomes, P bodies and stress granules, which could be investigated through fluorescent probing of candidate mRNAs. Also, a possible link to the new finding of co-translational mRNA degradation is detected (29). Since post-transcriptional regulation depends on mRNA motifs, the genes defined through this comparison could be used as a basis for further motif searches.

Concluding remarks

It is remarkable that the Hog1 MAPK is involved in such a wide array of processes in response to stress. Like the principal node of a vast network extending from the alert, available plasma membrane channels via the complicated nets of cell cycle regulators and carbohydrate metabolizers, it awakens the old nuclear bear of DNA for transcription and halts and turns the heavy machineries of translation and mRNA processing. In this thesis, the involvement of the Hog1 pathway in oxidative and osmotic stress and mRNA metabolism has been explored. It has been shown that the Hog1 pathway is involved in oxidative stress, but the question remains of what activates it, and if this explains why it is activated comparably late? The Hog1 pathway confers changes in polysomal association and mRNA stability, but the mechanism is not known. Hopefully closer examination of the large amounts of data will reveal patterns that could help in finding sequence motifs and effectors for these processes. Availability of large datasets concerning among other things polysomal association and mRNA stability, the opportunity arises of re-examining and directly comparing data from large scale experiments in order to shed new light on previous findings.

It is clear that MAPK pathways are central players during cellular stress of various kinds. Their involvement in multiple aspects of cellular metabolism lends them much importance when studying human disease that causes cellular stress, damage or any kind in inflammatory response. All this promises lots of interesting exploration and discovery in the future.

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