공학박사 학위논문

The regulation and function of Yak1 kinase in response to nutrient conditions in *Saccharomyces cerevisiae*

사카로마이세스 세레비지애에서 영양소 상태에 따른 Yak1 인산화 효소의 활성조절과 기능

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공과대학 화학생물공학부

이 베 드 로

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Abstract

The regulation and function of Yak1 kinase in response to nutrient conditions in *Saccharomyces cerevisiae*

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Yak1 is a member of an evolutionarily conserved family of Ser/Thr protein kinases known as dual-specificity Tyr phosphorylation-regulated kinases (DYRKs). Yak1 was originally identified as a growth antagonist which functions downstream of Ras/PKA signaling pathway in *Saccharomyces cerevisiae*. It has been known that Yak1 is phosphorylated by PKA (protein kinase A) *in vitro* and is translocated to the nucleus upon nutrient deprivation. However, the targets of Yak1

and the regulatory mechanisms for Yak1 activity and localization are not well understood. In this study, Yak1 was shown to function as an activator of stress responses upon glucose starvation through identifying its novel targets, Hsf1 and Msn2/4, and elucidating its PKA- and Bmh1/2-dependent regulatory mechanisms.

First, it was demonstrated that Yak1 kinase, which is under the negative control of PKA, activates both Hsf1 and Msn2 by phosphorylation when PKA activity is lowered by glucose depletion or by overexpressing Pde2 that hydrolyses cAMP. Hsf1 and Msn2/4 transcription factors play important roles in cellular homeostasis by activating gene expression in response to multiple stresses including heat shock, oxidative stress, and nutrient starvation. Yak1 directly phosphorylates Hsf1 *in vitro*, leading to the increase in DNA binding activity of Hsf1. Although Yak1 also phosphorylates Msn2 *in vitro*, it does not affect DNA binding activity or nuclear localization of Msn2 upon glucose depletion.

Second, it was shown that not only Yak1, but also Rim15, which is negatively regulated by PKA and TORC1 (Target of Ramapycin Complex 1), induces expression of Hsf1 target genes upon glucose depletion by both transcriptional activation and stabilization of the transcripts. Rim15 phosphorylates Hsf1 *in vitro*, suggesting that Rim15 might directly activate Hsf1. In addition, Igo1 and Igo2, which are

phosphorylated by Rim15, can stabilize the mRNA of *BTN2*, an Hsf1 target gene. Rim15 can phosphorylate Msn2, but not Gis1, *in vitro*, implying different mechanisms for the activation of these transcription factors.

Third, the role of PKA and Bmh1, a yeast 14-3-3 protein, in regulation of Yak1 was elucidated. PKA-dependent phosphorylation of Yak1 on Ser295 and two minor sites inhibits nuclear localization of Yak1. Intramolecular autophosphorylation on at least four Ser/Thr residues in the noncatalytic N-terminal domain is required for full kinase activity of Yak1. The most potent autophosphorylation site, Thr335, plays an essential role for Bmh1 binding in collaboration with a yet unidentified second binding site in the N-terminal domain. Bmh1 binding decreases the catalytic activity of Yak1 without affecting its subcellular localization. Considering the fact that Bmh1/2 binding to Yak1 coincides with PKA activity, such regulatory mechanisms might allow coordinated regulation of subcellular localization and kinase activity of Yak1 depending on glucose conditions.

Taken together, this study suggests important roles for Yak1 as well as Rim15 in ensuring cell survival under nutrient starvation conditions through induction of stress responses and growth arrest.

Keywords: autophosphorylation, Bmh1/2, Hsf1, Msn2/4, nutrient starvation, phosphorylation, PKA, Rim15, TOR, Yak1

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List of Abbreviations

cAMP : cyclic adenosine-monophosphate

CDK : cyclin-dependent kinase

CTA : C-termnial activation domain

DAPI : 4,6 diamidino-2-phenylindol

DBD : DNA-binding domain

DYRK(s) : dual-specificity Tyrosine phospohorylation-related kinases

EDTA : ethylenediaminetetraacetic acid

EGFP : enhanced green fluoresence protein

FKBP12 : FK506 binding protein

GFP : green fluoresence protein

GST : gluthathione-s-transferase

NES : nuclear localization signal

NLS : nuclear export signal

NTA : N-terminal activation domain

PAGE : polyacrylamide gel electrophoresis

PDS : post diauxic shift

PKA : protein kinase A

PKC : protein kinase C

PP2A : protein phosphatase 2A

SDS : sodium dodecyl sulfate

STRE : stress response element

TAR : transcription activation regions

TCA : trichloroacetic acid

TOR1/2 : target of rapamycin complex 1/2

Chapter 1.

Research Background and Objective

Including yeast, all living organisms have evolved complex and delicated signal transduction networks to ensure rapid and proper adaptation of cellular metabolism in response to the changes in nutrient condtions. This adjustment is sophisticatedly coordinated by a variety of singal trnasduction pathways, in which several nutrient-sensing kinases are involved in the regulation of cell growth and proliferation, and stress resistance. In yeast, there exist two major nutrient-sensing kinases, PKA (protein kinase A) and TOR (target of rapamycin), which control cell growth. Both signal pathways provide a variety of ways of cross-talk to regulate cell growth, showing functional overlaps including glucose fermentation, repression of genes involved in stress responses, inhibition of autophagy, and regulation of other cellular processes (De Virgilio & Loewith, 2006, Thevelein & de Winde, 1999, Santangelo, 2006). In addition, the PKA and TOR share several common mediators through different regulatory mechanisms including Yak1 (Martin *et al.*, 2004, Schmelzle *et al.*, 2004), and Rim15 kinases. It has been shown that both Yak1 and Rim15 are negatively regulated by both PKA and TOR pathways (Pedruzzi *et al.*, 2003, Reinders *et al.*, 1998).

It has been shown that Yak1, a member of an evolutionarily conserved family of Ser/Thr protein kinases known as dual-specificity Tyr phosphorylation-regulated kinases (DYRKs), which are

characterized by YXY motif in the activation loop in the catalytic domain (Kentrup *et al.*, 1996, Yoshida, 2008) can be directly phosphorylated by PKA *in vitro* and *in vivo*, suggesting that PKA might inhibit Yak1 through direct phosphorylation (Garrett *et al.*, 1991, Zappacosta *et al.*, 2002, Ptacek *et al.*, 2005). Furthermore, subcellular localization of Yak1 mainly depends on nutrients, as Yak1 accumulates in the nucleus upon either glucose starvation or rapamycin treatment (Moriya *et al.*, 2001, Martin et al., 2004, Schmelzle et al., 2004). In addition, Bmh1 and Bmh2, yeast 14-3-3 proteins, have been shown to be associated with Yak1 only in the presence of glucose, eliciting the speculation that Bmh1/2 might play a role in nuclear exclusion of Yak1 (Moriya et al., 2001). However, it has not yet been elucidated whether the regulation of nucleocytoplasmic shuttling of Yak1 is controlled by PKA and Bmh1/2.

Nutrient depletion leads to the inactivation of two major nutrient-sensing kinases, PKA and TOR, and starved cells show common characteristics such as G_1 cell cycle arrest, accumulation of storage carbohydrates (glycogen and trehalose), and increase in stress resistance (Herman, 2002). These phenotypes are closely related to stress responsive transcription factors Msn2 and Msn4 (Msn2/4), which are negatively regulated by PKA or TOR pathway and induce expression of target genes, which contains STRE (stress response

element) in their promoters (Martinez-Pastor et al., 1996, Santhanam et al., 2004, Smith et al., 1998). Furthermore, Msn2/4 can be activated by a variety of diverse environmental stresses including heat shock, osmotic stress, oxidative stress, and DNA damage through different signal transduction pathways.

In addition to Msn2/4, Hsf1, an evolutionally conserved heat shock transcription factor, plays an important role in cellular homeostasis in response to a variety of stress conditions including heat shock, oxidative stress and nutrient starvation. Although the mechanisms by which diverse stesses activate Hsf1 are not well understood, Hsf1 is thought to be regulated by stress-specific differential phosphorylation. It has been shown that Hsf1 activity is repressed by PKA, possibly by indirect inhibition of Hsf1 phosphorylation (Ferguson *et al.*, 2005). However, the regulatory mechanisms and the responsible kinase have not yet been elucidated.

The objective of this thesis is to understand the regulation and function of Yak1 kianse through the elucidation of its novel targets, Hsf1 and Msn2/4, involved in stress responses and its PKA- and 14-3-3 protein-dependent regulatory mechanisms.

Chapter 2.

Literature Review

2.1 Nutrient-sensing vs. stress response

Cells of all living organisms contain sophisticated mechanisms for sensing nutrients properly adapting to nutrient conditions through a wide range of signal transduction networks. It has been shown that a series of well-conserved nutrient-sensing protein kinases such as PKA and TOR perform key roles in regulating cell growth in response to nutrient availability. These nutrient-sensing kinases promote cell growth under high nutrient conditions while repressing stress responses. Upon nutrient depletion, inactivation of the nutrient-sensing kinases leads to the induction of stress responses through the activation of stress-responsive transcription factors. (Fig 2.1).

2.2 Nutrient sensing kinases

2.2.1 PKA

In *Saccharomyces cerevisiae*, cAMP-dependent protein kinase (PKA) , a highly conserved serine/threonine kinase, has three catalytic subunits (Tpk1, Tpk2, and Tpk3) which phosphorylate a variety of substrates involved in the metabolism of storage carbohydrates,

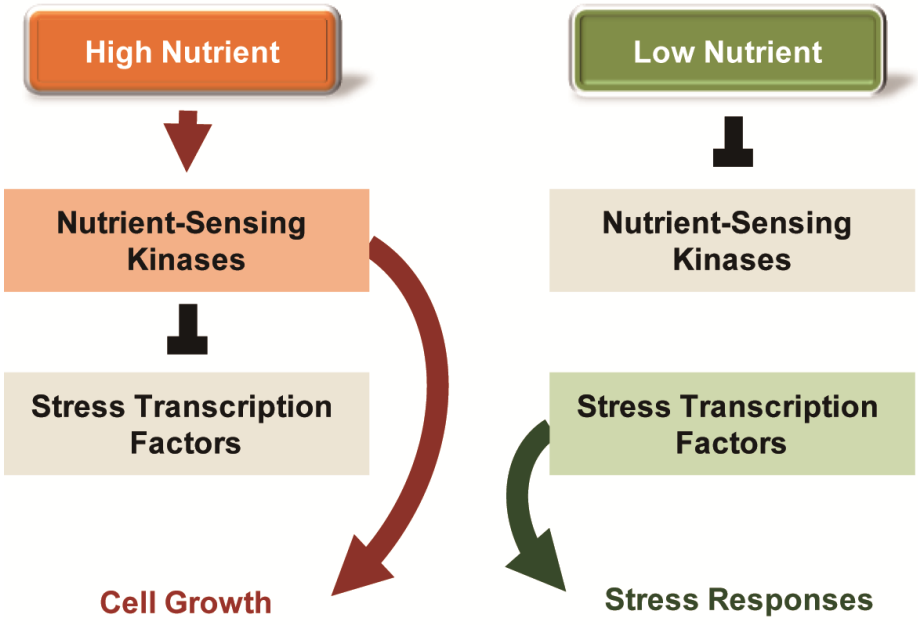


Figure 2.1 Nutrient-sensing vs. stress response

enzymes in glycolysis and gluconeogenesis, and transcription factors regulating stress response and ribosome biogenesis. The regulatory subunit of PKA is encoded by *BCY1* which binds and inhibits kinase activity of catalytic subunits (Cannon & Tatchell, 1987, Toda *et al.*, 1987). PKA is activated in response to glucose by two parallel signaling pathways; one involves Ras1 and Ras2 small GTPases and another involves Gpr1, a putative G protein coupled receptor, and its Gα protein Gpa2. Both pathways are converged to the activation of adenylate cyclase (Cyr1), resulting in the generation of cAMP that activates PKA by binding Bcy1 and releasing it from Tpks (Santangelo, 2006, Thevelein & de Winde, 1999, Johnson *et al.*, 1987). The level of cAMP in the cell is balanced by adenylyl cyclase-mediated catalyzed synthesis and phosphodiesterases, encoded by *PDE1* and *PDE2*, degradation of cAMP (Fig 2.1).

2.2.2 TOR

The highly conserved target of rapamycin (TOR) kinase is a central regulator of cell growth likewise PKA. Two homologous TOR kinases of *S. cerevisiae*, Tor1 and Tor2 exist in two complexes, TORC1 (including Tor1 or Tor2 and its subunits Kog1, Lst8, and Tco89) and TORC2 (Tor2

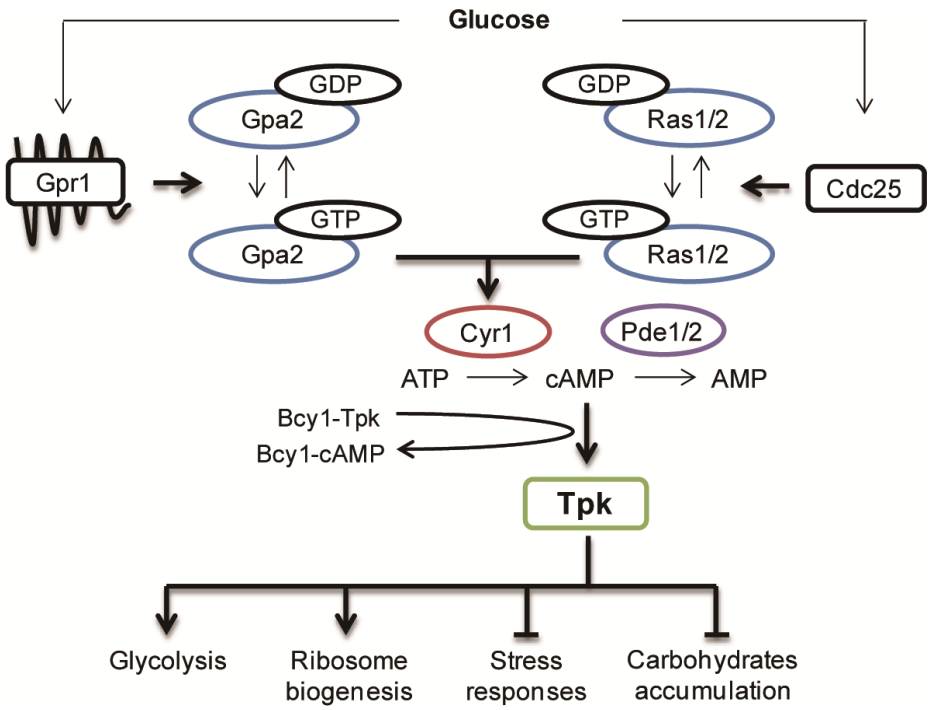


Figure 2.2 Diagram of yeast PKA signaling pathways.

and five other proteins, Avo1, Avo2, Avo3, Bit61, and Lst8), of which only TORC1 is inhibited by rapamycin, which in a complex with the prolyl isomerase FKBP12 binds to the complex and represses interaction with target substrates. Rapamycin treatment, mimics the effects of nitrogen starvation, leading to a number of responses including a decrease in ribosome biogenesis, activation of stress response gene transcription, and induction of autophagy. Furthermore, TORC1 not only regulates G1 cell cycle arrest and entry into G0-state, but also controls lifespan of yeast (Kaeberlein *et al.*, 2005). Therefore, TORC1 regulates cell proliferation and the proper transition between cell growth and quiescence. On the other hand, TORC2 controls the organization of the actin cytoskeleton, ceramide metabolism, and cell wall integrity (De Virgilio & Loewith, 2006, Aronova *et al.*, 2008, Cybulski & Hall, 2009) (Fig 2.2).

2.3 Stress-responsisve transcription factors

2.3.1 Hsf1 (heat shock factor1)

Heat shock factor1 (HSF1) is an evolutionarily conserved stress-

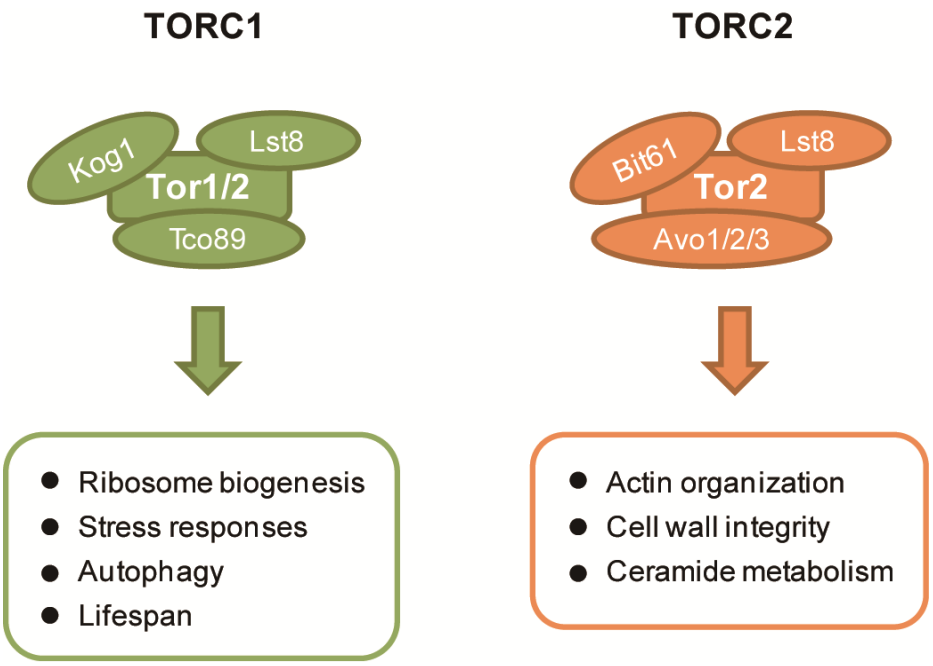


Figure 2.3 Functions of TORC1 and TORC2 in *S. cerevisiae*.

responsive transcriptional activator from yeast to humans that induces the expression of heat shock proteins, which maiantain cellular homeostasis both under normal and stress conditions. In addition to heat shock, Hsf1 can be activated by a variety of sresses including oxidative stress, glucose depletion, and ethanol stress (Hahn *et al.*, 2004, Yamamoto *et al.*, 2007, Tamai *et al.*, 1994).

The structure of Hsf1 contains a highly conserved helix-turn-helix DNA-binding domain (DBD), which binds to a 5 bp sequence nGAAn, and oligomerization domain, N-terminal activation domain (NTA), and carboxy-terminal activation domain (CTA) (Sorger, 1991) (Fig 2.4). Hsf1 forms homotrimeric complex and this form of Hsf1 binds to HSE (heat shock element). It has been shown that several types of HSE, which consists of the nGAAn unit, are recognized by Hsf1 in yeast. Heat shock elements are divided into three types: perfect-type HSE (nTTCnnGAAnnTTCn), gap-type HSE (nTTCnnGAAnnnnnnnGAAn), and step-type HSE (nTTCnnnnnnnTTCnnnnnnnTTCn) (Hashikawa *et al.*, 2007, Sakurai & Takemori, 2007).

2.3.2 Msn2 and Msn4

1) 40 – 147 : N-terminal activation domain (NTA)
2) 167 – 284 : DNA-binding domain (DBD)
3) 324 – 424 : Oligomerization domain (Oligo)
4) 584 – 783 : C-terminal activation domain (CTA)

Figure 2.4 The structure and function of Hsf1.

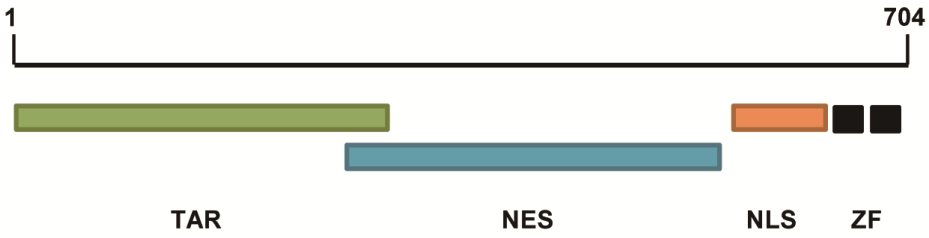
The Mns2 and Msn4 are transcriptional activators involved in the transcription of about 200 genes in *S.cerevisiae* (Causton *et al.*, 2001, Hasan *et al.*, 2002, Lallet *et al.*, 2006). Activation of Msn2/4 is triggered by diverse environmental stresses such as heat shock, osmotic stress, and nutrient starvation. Msn2/4 can induce expression of genes, containing STRE (stress response element) in their promoters (Hasan et al., 2002, Gasch *et al.*, 2000, Causton et al., 2001). Four domain of Msn2 has been elucidated. 1) N-terminal of Msn2 (1-303 a.a) - a transcription activation regions (TAR) (Boy-Marcotte *et al.*, 2006), 2) The amino acids 257-575 of Msn2 - nuclear export signal (NES) (Garmendia-Torres *et al.*, 2007), 3) The amino acids 576-648 of Msn2 - the nuclear localization signal (NLS) (Gorner *et al.*, 2002), and 4) C-terminal of Msn2 (642-698) - the DNA binding domains (DBD) (Boy-Marcotte *et al.*, 1998) (Fig 2.4).

To access to STRE sites, the subcellular localization of Msn2/4 are presicely controlled by several nutrient signaling pathways. So far, nuclear localization of Msn2/4 has been considered as the primary regulatory step for Msn2/4 activity. PKA-dependent phosphorylation of the nuclear localization signal (NLS) of Msn2 has been demonstrated to inhibit nuclear import of Msn2 (Gorner et al., 2002). On the other hand, PP2A phosphatase that is under the negative control of TOR has been shown to inhibit nuclear export of Msn2 upon inactivation of TOR

or in response to heat and osmotic stress (Beck & Hall, 1999, Santhanam et al., 2004). However, nuclear translocation seems not the only step for regulation of Msn2/4 activity. It has been shown that Msn2 activity can also be regulated at the level of DNA binding (Hirata *et al.*, 2003), transactivation (Boy-Marcotte et al., 2006), and degradation of nuclear Msn2 (Durchschlag *et al.*, 2004, Lallet *et al.*, 2004, Lallet et al., 2006).

2.4 Bmh1 and Bmh2

Members of the 14-3-3 family are highly conserved acidic proteins engaged in a wide range of biological functions by binding to phosphoserine or phosphothreonine residues in numerous target proteins (Fu *et al.*, 2000, Jones *et al.*, 1995, Wilker *et al.*, 2005). Two 14-3-3 binding motifs, RSXpS/pTXP (mode 1) and RXXXpS/pTXP (mode 2), have been identified based on the screening of phosphopeptide libraries (Muslin *et al.*, 1996, Yaffe, 2002), although some variations in binding motifs are identified in known 14-3-3 binding proteins (Johnson *et al.*, 2010). While yeast has only two isoforms, Bmh1 and Bmh2, mammals and plants contain at least 7 and 15 isoforms, respectively, which can form both homo- and heterodimers



1) 1 – 303 : Trans-Activation Region (TAR)
2) 257 – 575 : Nuclear Export Signal (NES)
3) 576 – 648 : Nuclear Localization Signal (NLS)
4) 642 – 698 : Zinc-fingers domain (ZF) (residues 647 - 665 and 676 - 698)

Figure 2.5 The stucture and function of Msn2.

(van Heusden & Steensma, 2006, Rosenquist *et al.*, 2001, Wang & Shakes, 1996). Each monomeric subunit of a 14-3-3 dimer can simultaneously bind to two binding motifs, leading to conformational changes in the binding partner, which in turn result in activation or inhibition of its enzymatic activity, or exposure or masking of functional motifs that regulate activity, cellular localization, stability, modification, or interaction with other proteins (Bridges & Moorhead, 2005, van Heusden & Steensma, 2006). In addition, if two binding motifs exist in separate proteins, 14-3-3 dimer can mediate interaction between two binding partners (Braselmann & McCormick, 1995). Proteomic analysis has revealed 271 yeast proteins interacting with Bmh1/2 in a phosphorylation-dependent manner (Kakiuchi *et al.*, 2007). However, the molecular consequences of Bmh1/2 binding have been elucidated for just a few proteins in *S. cerevisiae*. It has been shown that Bmh1/2 binding activates neutral trehalase (Nth1) activity (Panni *et al.*, 2008), facilitates Rad53 kinase function in DNA damage check point (Usui & Petrini, 2007), stimulates inhibitory activity of Mks1 in retrograde (RTG) signaling (Liu *et al.*, 2003), and retains Rim15, a protein kinase required for G_0 entry, in cytoplasm (Wanke *et al.*, 2005).

Chapter 3.

Experimental Procedures

3.1 Yeast strains, media, and culture conditions

Yeast strains used in this study are listed in Table 3.1. Deletion mutants *yak1Δsnf1Δ* and *yak1Δ msn2/4Δ* were generated by integration of the PCR-amplified *yak1Δ::KanMX6* cassette into BY4741 *snf1Δ* (Hahn & Thiele, 2004) and *yak1Δ,* and *rim15Δ* were also generated by integration of the PCR-amplified *yak1Δ::LoxP* or *rim15Δ::LoxP* cassette into BY4741 strain, and W303-1A *msn2/4Δ* strain (Estruch & Carlson, 1993) respectively. L40-Ura was used for yeast two-hybrid assay. DTY185/yak1Δ strain was generated by integration of the PCR-amplified *yak1Δ::URA3* cassette into DTY185, a *PEP4* deletion strain. Yeast cells were grown in YPD medium (1% yeast extract, 2% bactopeptone, 2% dextrose) or synthetic complete (SC) medium lacking appropriate amino acids. For stress induction, cells were grown in YPD medium to OD_{600} of 1-1.5 and then shifted to YP medium without glucose, heat shocked at 39 ^{o}C, or treated with 200 nM rapamycin.

Table 3.1 *S. cerevisiae* strains used in this study

Strains	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
BY4741 *yak1Δ*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yak1Δ::KanMX4*	EUROSCARF
BY4741 *yak1Δ*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yak1Δ::LoxP*	This study
BY4741 *snf1Δ*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1Δ*	(Hahn & Thiele, 2004)
BY4741 *yak1Δ snf1Δ*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1Δ yak1Δ::KanMX6*	This study
BY4741 *rim15Δ*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rim15Δ::LoxP*	This study
BY4741 *rim15Δ rim15Δ*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yak1Δ rim15Δ::LoxP*	This study
W303-1A *msn2/4Δ*	*MATa ade2-1 can1-100, leu2-3,-112 trp1-1 ura3-1 msn2::HIS3, msn4::TRP1*	(Estruch & Carlson, 1993)
W303-1A *yak1Δmsn2/4Δ*	*MATa ade2-1 can1-100, leu2-3,-112 trp1-1 ura3-1 msn2::HIS3, msn4::TRP1 yak1Δ::KanMX6*	This study
EJ758	*MATa his3-Δ200*, *leu2-3,112*, *ura3-52*, *pep4::URA3*	(Martzen *et al.*, 1999)
L40-Ura	*MATa his3-Δ200 trp1-910 leu2-3,112 ade2 lys::(lexAop)$_4$-HIS3 ura3::(lexAop)$_8$-lacZ*	
DTY185/*yak1Δ*	*MATa ade2 leu2-3,112 his3 trp1 ura3 pep4::TRP1 yak1Δ::URA3*	This study
CDV288-12A	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 igo1Δ::KanMX4 igo2Δ::KanMX4*	(Talarek *et al.*, 2010)

3.2 Plasmids

3.2.1 *E.coli* expression plasmids

Plasmids pGEX4T-1-*YAK1* and pGEX4T-1-*YAK1*^{K398R} were generated by cloning PCR product containing the wild type or mutant *YAK1* ORF into the SmaI and XhoI sites of pGEX4T-1. For the pGEX4T-1-*YAK1*^{K398R}, pRS416-*YAK1*^{K398R} was used as a PCR template. Other expression vectors for GST-fusion proteins of Yak1 derivatives were constructed by cloning of the PCR products into SmaI and XhoI sites of pGEX4T-1 vector. The expression vectors for GST-Hsf1 and GST-Hsf1 (1-583) were described previously (Hahn & Thiele, 2004). The expression vectors for various length of GST-fused Hsf1 (1-155, 1-180, 1-200, 1-230, 1-260, and 147-583) were generated by cloning the PCR products into the SmaI and EcoRI sites of pGEX-3x. The expression vectors for GST-Hsf1 (1-260) and GST-Hsf1 (261-833) were generated by cloning the PCR products into EcoRI and XhoI sites of pGEX4T-1. The expression vectors for full-length or truncated derivatives of GST-Msn2 were constructed by cloning of the PCR-amplified SmaI-NotI fragments of Msn2 (1-704), Msn2 (1-400), and Msn2 (401-704) into pGEX4T-1 vector. The expression vectors for truncated derivatives of

GST-Ifh1 were constructed by cloning of the PCR-amplified SmaI-XhoI fragments of Ifh1 (1-400), Ifh1 (401-800) into pGEX4T-1 vector. Site specific mutagenesis using overlapping PCR was performed to generate pGEX4T-1-*IFH(401-800)*^{S522A}. The expression vector for GST-Tpk1 was generated by cloning the *TPK1* ORF into EcoRI and BamHI sites of pGEX4T-1. The expression vector for GST-Gis1 was generated by cloning a 2651 bp PCR fragment containing *GIS1* ORF into SmaI and XhoI sites of pGEX4T-1. The expression vectors for GST-Igo1 (pLC1092) (Talarek et al., 2010) and GST-Rim15 (pCDV487) (Pedruzzi et al., 2003)were provided by Dr. C. D. Virgilio (University of Fribourg, Switzerland). The expression vector for His_6-fused Bmh1 was generated by inserting *BMH1* ORF into BamHI and XhoI sites of pET28b(+). All the site specific mutageneses were performed using overlapping PCR and the mutated sequences were verified by DNA sequencing.

3.2.2 Yeast expression plasmids

Plasmid pRS416GDP-*YAK1* was generated by cloning a 2.4 kb PCR fragment containing *YAK1* ORF into the HindIII and XhoI sites of pRS416GPD. Site specific mutagenesis using overlapping PCR was

performed to generate p416GDP-*YAK1*^{K398R}. The GPD promoters of pRS416GDP-*YAK1* and p416GDP-*YAK1*^{K398R} were removed by cutting the plasmids with SacI and SpeI, and replaced by 500 bp *YAK1* promoter, generating pRS416-*YAK1* and pRS416-*YAK1*^{K398R}, respectively. Plasmid pRS415GDP-*EGFP* was generated by cloning a 0.75 kb PCR fragment containing *EGFP* ORF into SpeI and HindIII sites of pRS415GPD. The GPD promoter of pRS415GPD-*EGFP* was removed by cutting the plasmids with SacI and SpeI, and replaced by 500 bp *YAK1* promoter, generating pRS415-YAK1p-*EGFP*. And then, a 2.4 kb PCR fragment containing *YAK1* ORF was cloned into HindIII and XhoI sites of pRS415-YAK1p-*EGFP*, resulting in pRS415-YAK1p-*EGFP*-*YAK1*. For two-hybrid analysis, all the *YAK1* truncated derivatives and mutants were amplified by PCR and then cloned into the SmaI and PstI sites of pBTM116 to express them as LexA DBD-fusion proteins. *BMH1* ORF was cloned into BamHI and NotI sites of pVP16 to generate plasmid expressing VP16-Bmh1. The plasmid YEplac*PDE2* and pADH1-*MSN2-GFP* (Gorner *et al.*, 1998) was provided by Dr. Pual Herman and Dr. Christoph Schüller, respectively. The HSE-*CYC1-lacZ* reporter plasmids containing 6P, 3P, and gap type HSE were provided by Dr. Hiroshi Sakurai (Hashikawa *et al.*, 2006). Plasmid pRS426GAL1-*GST* was generated by cloning a 0.75 kb PCR fragment containing GST from pGEX4T-1 into SpeI and HindIII

sites of pRS426GAL1. And then, pRS426GAL1-*GST-RIM15*$^{K823Y/C1176Y}$ was generated by homologous recombination.

3.3 Protein purifications

In order to purify GST- or His-fusion proteins from *E. coli*, Rosetta gami2 (DE3)pLysS strain was transformed with various expression vectors, and the proteins were induced with 1 mM IPTG for 3 h. GST-fusion proteins were purified using glutathione-agarose and His-fusion proteins were purified using Ni^{2+}-agarose. The purified proteins were stored at -70 ^{o}C after dialysis against dialysis buffer containing 50 mM Tris-HCl (pH 8.0) and 10% glycerol.

In order to purify GST-Rim15 proteins from *S. cerevisiae*, Expression vector for GST-Rim15 (pCDV487) or GST-Rim15$^{K823Y/C1176Y}$ (pRS426GAL1-*GST-RIM15*$^{K823Y/C1176Y}$) was transformed into *rim15Δyak1Δ* strain. The transformants were cultured overnight in SC-Ura medium containing 2 % raffinose, and then transferred to SC-Ura medium containing 4 % galactose for 4 h. GST-fusion proteins were purified from cell extracts using glutathione-agarose (Novagen), and stored at -70 ^{o}C.

3.4 *In vitro* pull-down assays

For His pull-down assays to detect interaction between Bmh1 and Yak1, 10 μg of His-Bmh1 proteins were immobilized on Ni^{2+}-NTA agarose that had been previously equilibrated in IP150 buffer [50 mM HEPES(pH 7.5), 150 mM NaCl, 0.5% NP-40, 1 mM EDTA , and 0.01% protease inhibitor cocktail] containing 50 mM Imidazole. The immobilized His-Bmh1 proteins were incubated with soluble yeast cell lysates or GST-Yak1 proteins at 4 ^{o}C for 1 h. For GST-pull down assays, 5 μg of His-Bmh1 proteins were incubated overnight at 4 C^{o} with 5 μg of GST-Yak1 immobilized on glutathione-agarose in IP150 buffer. After protein binding, the Ni^{2+}-NTA agarose or glutathione-agarose beads were washed three times with IP150 buffer and the bound proteins were eluted by boiling in sample buffer for 10 min. Samples were resolved by 8 or 10% SDS-PAGE and proteins were detected by either immunoblotting or silver staining.

3.5 *In vitro* protein kinase assays

In order to screen kinases phosphorylating Hsf1, an array of 136 *S.*

cerevisiae EJ758 strains each containing an expression vector for GST-fused kinase was generated from the GST-ORF library of yeast genome. GST-kinases purified in 9 pools of 15 or 16 strains each were bound to glutathione-agarose, and *in vitro* kinase assays were performed using GST-Hsf1 purified from *E. coli* as a substrate as described previously. GST-kinases in the pools of having Hsf1 phosphorylation activity were individually purified and assayed for Hsf1 phosphorylation to identify the specific kinases. In order to purify GST fusion proteins from *E. coli*, BL21 (DE3) strain was transformed with various expression vectors, and the proteins were induced with 1 mM IPTG for 3 h, purified using glutathione-agarose, and stored at -70 ^{o}C after dialysis against dialysis buffer [50 mM Tris HCl (pH 8.0), 1 mM DTT, and 20% glycerol] . Kinase assays using GST-Yak1 purified from *E. coli* were performed by incubating 2 μg of GST-Yak1 with 1 to 5 μg of GST-Hsf1, GST-Msn2(1-400), GST-Msn2(401-704), GST-Ifh1(1-400), GST-Ifh1(401-800), and GST-Ifh1(401-800)^{S522A} purified from *E. coli* in 25 μl reaction buffer containing 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 μM ATP, and 5 μCi of [γ-^{32}P]-ATP at room temperature for 30 min. Reactions were terminated by boiling in SDS-PAGE sample buffer. The reaction products were separated by SDS-PAGE and the phosphorylated proteins were detected by phosphorimager analysis.

For *in vitro* kinase assays using either GST-Tpk1 or GST-Yak1 and its derivatives, 3 or 5 μg of kinases were incubated with 1 or 3 μg of various substrate proteins for 1 h at room temperature in 30 μl of reaction buffer containing 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 μM ATP, and 2.5 μCi of [γ-^{32}P]-ATP. Kinase reactions were terminated by boiling in SDS-PAGE sample buffer. The reaction products were separated by SDS-PAGE and the phosphorylated proteins were detected by phosphorimager analysis.

For kinase assays using GST-Rim15 purified from *S.cerevisiae* or GST-Yak1 purified from *E. coli* were performed by incubating 0.1 μg of kinase with 1 to 5 μg of GST-Igo1, GST-Hsf1, GST-Gis1, GST-Msn2 purified from *E. coli* in 30 μl reaction buffer containing 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 μM ATP, and 5 μCi of [γ-^{32}P]-ATP at room temperature for 60 min. Reactions were terminated by boiling in SDS-PAGE sample buffer. The reaction products were separated by SDS-PAGE and the phosphorylated proteins were detected by phosphorimager analysis.

3.6 Substrate specificty screening of kinases

In the screening of Ser/Thr kinase substrate specificities, Yak1 kinase

was applied to a serine kinase reaction buffer (50mM Tris-HCl pH 7.4, 10mM $MgCl_2$) and incubated with peptide coupled resin beads for 4 hrs at 30℃, respectively. The beads were mixed with biotin conjugated anti-phosphoserine antibody (5 g/ml) and were incubated for 1.5 h. The beads were mixed with neutravidin-AP(12 g/ml) solution and were incubated for 20 min. Finally, a standard alkaline phosphatase substrate mixture (NBT; nitro-blue tetrazolium chloride /BCIP; 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt) in 50mM PBS was added to the beads and reaction solution was incubated for 30min. Phosphorylated beads were identified by their dark colors using a low magnification microscope (X40). The selected beads were irradiated by UV (spot light source, 365 nm, Lightningcure 2000, hamamatsum Photonic K) for 30 min to cleave the photocleavable linker. The peptide sequences of the each selected bead were analyzed by MALDI-TOF MS. MALDI-TOF mass spectrometric analysis was performed with a Bruker Datonics Biflex IV time of flight mass spectrometry (Bruker, Germany). Each spectrum was the average result of 100 laser shots and the average value of measurements was acquired from Bruker X-TOF 5.1.1. and Biotools 2.1 programs.

3.7 Elcetrophoretic mobility shift assay (EMSA)

Before determining the binding affinity of GST-Hsf1 to HSE, 50 μg of GST-Hsf1 were phosphorylated by 15 μg of GST-Yak1 purified from *E.coli* in 90 μl reaction buffer containing 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, and 100 μM ATP at 30 ^{o}C for 1 h 30 min. As a control, GST-Hsf1 was incubated with GST-Yak1^{K398R} instead of GST-Yak1. Increasing amount of kinase reaction mixture was then incubated with 0.2 ng of ^{32}P-end-labeled annealed double strand DNA fragment containing three HSE units (5'-TCGACTTCTAGAAGCTTCCACTAATTAGTGC-3',HSE units are underlined), five HSE units (5'-TCGACTTCTAGAAGCTTCCAGAAATTTCTGG-3') or gap type HSE (5'-TCGACTTCTAGAAGCTAGGAGAAATTAGTGC-3') in 20 μl of binding buffer [20 mM HEPES-KOH (pH 7.5), 2 mM $MgCl_2$, 0.5 mM EDTA, 25 mM Potassium acetate, 0.05% Nonidet P-40, 0.4 μg of poly(dI-dC), and 5% glycerol] at room temperature for 15 min. The samples were electrophoresed on 6% polyacrylamide gel in 0.5x TBE (45 mM Tris-borate and 1 mM EDTA) at room temperature and then subjected to phosphorimager analysis. Fractions of bound DNA were calculated through the equation $R = I_b/(I_b + I_f)$, where I_b and I_f are the intensities of HSE-bound and free probe bands, respectively. The equilibrium dissociation constant (K_d) was estimated from the GST-

Hsf1 concentration required for half-maximal binding of Hsf1 to each probe DNA.

To determine the effect of Yak1-dependent phosphorylation on Msn2 DNA binding activity, 25 μg of GST-Msn2 (401-704) was phosphorylated by 10 μg of GST-Yak1 purified from *E.coli* or incubated with GST-Yak1^{K398R} in 80 μl reaction buffer containing 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, and 100 μM ATP at 30 ^{o}C for 90 min. Increasing amount of the kinase reaction mixture was then incubated with 0.2 ng of ^{32}P-end-labeled DNA fragment containing -400 to -300 region of *CTT1* promoter in 20 μl of binding buffer [10 mM Tris-HCl (pH 8.0), 33.3 mM NaCl, 1mM DTT, 0.005% Nonidet P-40, 0.4 μg of poly(dI-dC), and 5% glycerol] at room temperature for 15 min. The samples were electrophoresed on 6% polyacrylamide gel in 0.5x TB (45 mM Tris-borate) at room temperature and then subjected to phosphorimager analysis.

3.8 RT-PCR

Total RNA was isolated from yeast cells and the relative amount of specific mRNA was determined by semi-quantitative RT-PCR. Briefly, 1 μg of the total RNA was subjected to reverse transcription in a 20 μl

reaction mixture containing 200U of myeloblastosis virus reverse transcriptase (M-biotech) and 0.1 μg of oligo-(dT) at 42 °C for 55 min. 1 μl of the reaction mixture was then subjected to PCR reaction with gene-specific primers. PCR was performed with 15 to 20 cycles of 95 °C for 10 sec, 55 °C for 30 sec, and 72 °C for 30 sec. The amount of PCR products were analyzed by using Image J image processing program (NIH).

For quantitative RT-PCR analysis, cDNAs were subsequently quantified by real-time PCR (LightCycler480) using SYBR green master mix (Roche). The reactions were performed with 1 cycle at 95 °C for 5 min, followed by 45 repeated cycles at 95 °c for 20 s, 60 °C for 20 s, and 72 °C for 30 s. The *ACT1* transcripts were used as a control for the quantitative PCR. qRT-PCR primers amplified a 200-bp region of *BTN2* BTN2F:5'-AGCAATTCTGGTTCAGCAGAAAG-3'; BTN2R:5'-TTATATCTCCTCAATAATAGAGTTT-3'), a 201-bp region of *HSP26* (HSP26F:5'-AAGGTCAAGGAGAGCAGCTCTG-3'; HSP26R:5'-TTAGTTACCCCACGATTCTTGAG-3'), a 202-bp region of *CTT1* (CTT1F:5'- CAGAAGAAATTATTCGTTCATAACG-3'; CTT1R:5'-TTAATTGGCACTTGCAATGGACC-3'), and a 106-bp region of *ACT1* (ACT1F:5'-GCCGAAAGAATGCAAAAGGA-3'; ACT1R:5'-TAGAACCACCAATCCAGACGG-3')

3.9 Immunoblotting

For detection of Hsf1 protein whole yeast cells were precipitated with a final concentration of 5% TCA (trichloroacetic acid), the pellets were washed twice with acetone, dried, resuspended in 50 μl of boiling buffer [50mM Tris (pH 7.5), 1mM EDTA, and 1% SDS], and broken by vortexing in the presence of glass beads (sigma). The samples were boiled after addition of SDS sample buffer, and then subjected to 6% SDS-PAGE. Hsf1 protein was detected by immunoblotting with anti-HSF antibody.

3.10 Yeast two-hybrid assays

Yeast two-hybrid assays were performed by transformation of S. cerevisiae L40-Ura cells with plasmids expressing VP16- and LexA DBD-fusion proteins. Cells harboring the appropriate constructs were grown in liquid selective medium (SC-Leu-Trp) and harvested from log-phase cultures. After dilution of the cells with water to OD_{600} of 1 and 0.1, 5 μl of the cells were spotted on a control plate (SC-Leu-Trp) and

a selective plate (SC-Leu-Trp-His containing 50 mM 3-AT), and incubated at 30 C^{o} for 2 to 3 days.

3.11 Fluorescence microscopy

BY4741 and *yak1Δ* cells harboring pADH1-*MSN2-GFP* were grown to early exponential phase and cellular localization of Msn2-GFP was monitored by fluorescence microscopy (Nikon ECLIPSE 80i). To localize EGFP-Yak1 and its derivatives, DTY185/*yak1Δ* cells containing the plasmids were grown in a selective SC medium containing 4% glucose until early exponential phase. For glucose starvation, cells were shifted to a medium without glucose for 1 h. Harvested cells were fixed with 3.7% paraformaldehyde and 0.05% Triton X-100 for 20 min. After washing the cells for three times with 1xPBS, cellular localization of EGFP signals were monitored by fluorescence microscopy (Nikon ECLIPSE Ti-U). Localization of nuclei was detected by staining of the DNA with 2.5 μg/ml of 4, 6 diamidino-2-phenylindol (DAPI). Quantification of the ratios of fluorescence intensity (nucleus/cytoplasm) was analysed by using Image J image processing program (NIH).

Chapter 4.

Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4

4.1. Introduction

Nutrient starvation leads to the inhibition of PKA and TORC1, and nutrient starved cells show common characteristics such as G1 cell cycle arrest, accumulation of storage carbohydrates (glycogen and trehalose), and increase in stress resistance (Herman, 2002). These phenotypes are closely related to the roles of functionally redundant transcription factors Msn2 and Msn4 (Msn2/4), activated by inactivation of PKA or TOR pathway. Not only nutrient starvation, but also diverse environmental stresses including heat shock, osmotic stress, oxidative stress and DNA damage can activate Msn2/4 through different signal transduction pathways.

The role for Msn2/4 in growth inhibition is supported by the fact that deletion of both *MSN2* and *MSN4* can suppress the growth defect of a strain lacking all the three catalytic subunits of PKA (Smith et al., 1998). Deletion of Yak1 kinase can also suppress the PKA-deficient growth defect although not as effectively as the deletion of *MSN2* and *MSN4* (Smith et al., 1998, Garrett & Broach, 1989), suggesting the role for Yak1 as a growth antagonist. Yak1 is negatively regulated by PKA supposedly by direct phosphorylation (Garrett et al., 1991), and is translocated to the nucleus upon glucose depletion or rapamycin

treatment (Moriya et al., 2001, Martin et al., 2004, Schmelzle et al., 2004). So far, several substrates of Yak1 have been identified, including Bcy1, a regulatory subunit of PKA (Griffioen *et al.*, 2001), Pop2, a component of Ccr4-Not complex that regulates 3' to 5' mRNA decay (Moriya et al., 2001, Daugeron *et al.*, 2001), Dcs1 involved in mRNA decapping (Malys *et al.*, 2004), and Crf1, which acts a transcriptional co-repressor for ribosomal protein genes (Martin et al., 2004).

In addition to Msn2/4, Hsf1, heat shock transcription factor, plays a central role in cellular homeostasis in response to a variety of stress conditions including heat shock, oxidative stress, and nutrient starvation. In *S. cerevisiae,* Hsf1 is essential for cell survival, and unlike Msn2/4, Hsf1 is always in the nucleus. Depending on the architecture of the Hsf1 binding site known as heat shock element (HSE), Hsf1 is engaged in both constitutive and stress-inducible DNA binding, regulating expression of genes involved in protein folding and degradation, and other broad range of biological functions (Hahn *et al.*, 2004). Although the mechanisms by which many diverse stresses activate Hsf1 are not well understood, Hsf1 is thought to be regulated by stress-specific differential phosphorylation. Previously, it has been shown that Snf1-dependent phosphorylation can activate Hsf1 upon glucose starvation through enhancing DNA binding activity, without

affecting activation of Hsf1 by heat shock (Hahn & Thiele, 2004). However, induction of the Hsf1 targets was still observed in a Snf1-independent manner under the conditions of acute glucose starvation, suggesting the presence of other pathways for Hsf1 activation. PKA has been shown to repress Hsf1 activity, possibly by indirect inhibition of Hsf1 phosphorylation (Ferguson et al., 2005). However, the regulatory mechanisms and the responsible kinase have poorly understood.

In this chapter, it has been demonstrated that Yak1 kinase phosphoyrlates and activates Hsf1 under the condtions of low PKA activity. Furthermore, it has been shown that Yak1, whose expression is induced by Msn2/4, can also activate Msn2 possibly by direct phosphorylation. These results suggest an important role for Yak1 in mediating PKA-dependent inhibition of stress responses.

4.2 Screening of protein kinases phosphorylating Hsf1 *in vitro*

In order to investigate the role for differential phosphorylation in regulation of Hsf1 activity, yeast protein kinases were screend for the

phosphorylation of Hsf1 *in vitro*. 136 putative protein kinases in *S. cerevisiae* genome (Hunter & Plowman, 1997) were expressed as GST-fusion proteins in *S. cerevisiae* EJ758 strain (Martzen *et al.*, 1999), and was screened for their activity toward phosphorylation of Hsf1 *in vitro*. The identified kinases include Snf1, Cmk1, Cmk2, Kns1, and Yak1. The role for Snf1 in phosphorylation and activation of Hsf1 under glucose starvation conditions has been reported previously (Hahn & Thiele, 2004). Cmk1 and Cmk2 kinases are calcium/calmodulin-dependent protein kinases and their mammalian homologue CaMKII has been shown to phosphorylate and activate human Hsf1 (Holmberg *et al.*, 2001). Kns1 is a member of LAMMER kinase family, and its fission yeast homologue Lkh1 has been shown to be involved in filamentous growth, flocculation, and oxidative stress response (Kang *et al.*, 2007, Kim *et al.*, 2001). The roles for Cmk1, Cmk2, and Kns1 in regulation of yeast Hsf1 still remain to be elucidated. This thesis will be focused on the characterization of the Yak1 kinase.

4.3 Yak1 is involved in growth phase-dependent phosphorylation of Hsf1 and activation of Hsf1 and Msn2/4

Yak1 kinase is negatively regulated by PKA and acts as an antagonist for cell growth (Garrett et al., 1991). It has been shown that both Msn2/4-dependent expression of *YAK1* and Yak1 activity increase in post exponential phase of cell growth (Moriya et al., 2001, Smith et al., 1998). Therefore, in order to investigate whether Yak1 is involved in the phosphorylation of Hsf1 *in vivo*, Hsf1 phosphorylation patternsin wild type and *YAK1* deletion mutant were compared during cell growth. The electrophoretic mobility of Hsf1 was gradually reduced in both wild type and *yak1Δ* as cells were grown from early (OD_{600} of 1) to late exponential phase (OD_{600} of 7 and 9), reflecting increase in Hsf1 phosphorylation in response to growth-related changes in the medium (Fig. 4.1A and B). It was confirmed that the mobility shift was due to phosphorylation by treating the cell extracts with λ phosphatase, which led to the Hsf1 bands with the same increased mobility in both wild type and *yak1Δ* independent of the growth phase (data not shown). Although *yak1Δ* showed no growth defect as previously reported (Garrett & Broach, 1989, Garrett et al., 1991), *yak1Δ* showed reduced levels of Hsf1 phosphorylation compared with wild type especially at late exponential phase, suggesting that Yak1 is involved in growth phase-dependent phosphorylation of Hsf1. However, even in *yak1Δ,*

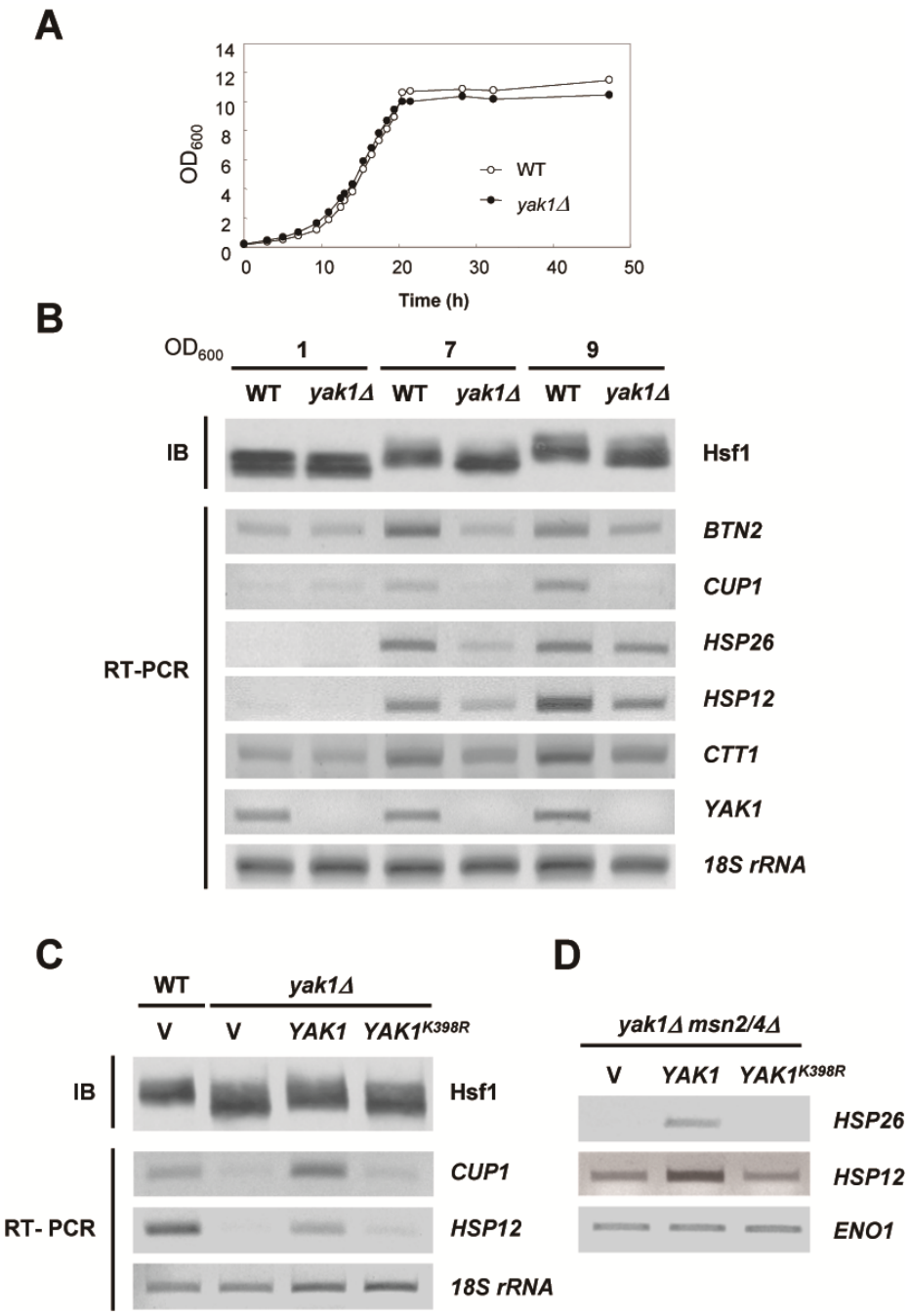

A
OD600
Time (h)
WT
yak1Δ
B
OD600
1
7
9
WT
yak1Δ
IB
Hsf1
RT-PCR
BTN2
CUP1
HSP26
HSP12
CTT1
YAK1
18S rRNA
C
WT
yak1Δ
V
YAK1
YAK1K398R
IB
Hsf1
RT- PCR
CUP1
HSP12
18S rRNA
D
yak1Δ msn2/4Δ
HSP26
HSP12
ENO1

Figure 4.1 Growth phase-dependent phosphorylation of Hsf1 and activation of Hsf1 and Msn2/4 are dependent on Yak1. (A) Growth curves of BY4741 (WT) and *yak1Δ* cells grown in SC (synthetic complete) medium. (B) Yak1-dependent Hsf1 phosphorylation and activation of the Hsf1- and Msn2/4-regulated genes during growth. At the indicated OD_{600}, cells were withdrawn to prepare cell extracts and RNA. Phosphorylation of Hsf1 was detected by immunoblotting (IB) with antibody against Hsf1 and mRNA levels were detected by RT-PCR. *18S rRNA* was used as a control. (C) Restoration of phosphorylation and activation of Hsf1 by expression of the *YAK1* gene in *yak1Δ* strain. BY4741 (WT) cells containing empty vector (V) and *yak1Δ* cells containing empty vector, pRS416-*YAK1*, or pRS416-$YAK1^{K398R}$ were grown in SC-Ura medium until late exponential phase, and Hsf1 phosphorylation and mRNA expression levels of the Hsf1 target genes were monitored. (D) Yak1-dependent expression of *HSP26* and *HSP12* in *msn2/4Δ* mutant. The *yak1Δ msn2/4Δ* triple mutant cells carrying the indicated plasmids were grown in SC-Ura medium to late exponential phase, and mRNA expression levels were detected by RT-PCR. *ENO1* was used as a control.

Hsf1 phosphorylation was gradually increased during growth, implying the presence of other kinases phosphorylating Hsf1.

To examine the effect of Hsf1 phosphorylation on Hsf1 activity, mRNA expression levels of Hsf1 target genes by RT-PCR was detected (Fig. 1B). While *BTN2* and *CUP1* genes are regulated by Hsf1 only, *HSP12* and *HSP26* are regulated by Msn2/4 as well as Hsf1. In wild type, all the Hsf1 target genes showed increased expression at late exponential phase, but their expression levels were lower in *yak1Δ* than wild type, exhibiting nice correlation with the levels of Hsf1 phosphorylation. These results clearly suggest that Yak1-dependent phosphorylation activates Hsf1. Msn2/4-dependent expression of *CTT1* and *YAK1* was also increased during growth as expected (Fig. 4.1B). Interestingly, *CTT1* expression levels were also decreased in *yak1Δ* compared with wild type, suggesting a possible implication of Yak1 in regulation of Msn2/4 as well as Hsf1.

The role for Yak1 in Hsf1 activation was further confirmed by introducing a plasmid harboring *YAK1* or a catalytically inactive *YAK1*^{K398R} mutant gene into *yak1Δ* strain. Hsf1 phosphorylation and expression of the Hsf1 target genes were restored in *yak1Δ* at late exponential phase by expression of Yak1, but not by Yak1^{K398R} kinase-dead mutant, confirming the requirement of Yak1 kinase activity for

Hsf1 activation (Fig. 4.1C). To rule out the effect of Msn2/4 in expression of *HSP12* and *HSP26*, a triple deletion mutant lacking *YAK1* in the *msn2/4Δ* mutant background was generated. Expression of active Yak1 in this mutant restored expression of *HSP12* and *HSP26* at late exponential phase, further supporting the Yak1-dependent activation of Hsf1 (Fig. 4.1D).

4.4 Yak1 is involved in activation of Hsf1 and Msn2/4 upon acute glucose depletion

The Yak1-dependent activation of Hsf1 and Msn2/4 at late exponential phase suggests that Yak1 might regulate Hsf1 and Msn2/4 in response to glucose limitation. Therefore, it was tested whether Yak1 is involved in induction of the target genes of Hsf1 and Msn2/4 under the conditions of acute glucose depletion. When cells grown in the presence of 2% glucose were shifted to a medium without glucose for 0.5 and 1 h, wild type cells showed greater induction of Hsf1 phosphorylation then *yak1Δ*, indicating that Yak1 is responsible for Hsf1 phosphorylation in response to glucose depletion (Fig. 4.2A). Expression levels of *BTN2* reflected the patterns of Hsf1 phosphorylation, exhibiting reduced levels of induction in *yak1Δ* than

wild type (Fig. 4.2A). However, the fact that *BTN2* induction was not completely abolished in *yak1Δ* implies that Yak1 is not the only kinase involved in Hsf1 activation upon glucose depletion. Induction of *CTT1* was also reduced in *yak1Δ*, which again supports the role for Yak1 in regulation of Msn2/4 activity. Induction of *HSP26*, which are regulated by both Hsf1 and Msn2/4, showed the most dramatic reduction in *yak1Δ*, which might be caused by a combinatorial effect of Yak1 on Hsf1 and Msn2/4.

Previously, it has been shown that Snf1 is responsible for activation of Hsf1 under the conditions of prolonged glucose starvation in the presence of low (0.05 %) glucose, but Snf1 is almost dispensable for Hsf1 activation upon acute glucose depletion. In agreement with the previous results, unlike *yak1Δ* which reproducibly showed reduced level of *BTN2* induction, deletion of *SNF1* did not affect induction of *BTN2* upon acute glucose depletion (Fig. 4.2B). The double deletion mutant *yak1Δsnf1Δ* still showed *BTN2* induction, implying the presence of Hsf1 activation mechanisms independent of Yak1 and Snf1. The higher induction levels of *HSP26* and *CTT1* in *snf1Δ* than wild type agree with the proposed inhibitory role for Snf1 in Msn2/4 activity (Hahn & Thiele, 2004, De Wever *et al.*, 2005, Sanz *et al.*, 2000). The increased levels of *HSP26* and *CTT1* in *snf1Δ* were

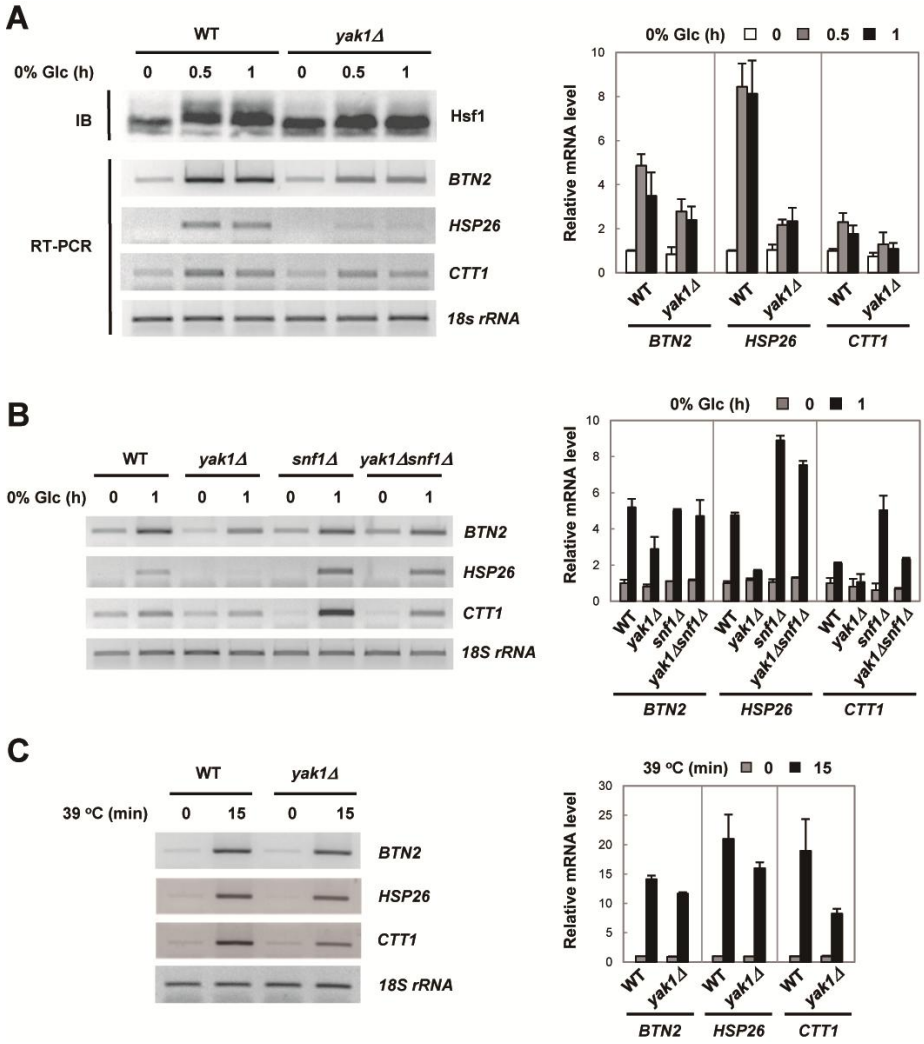

A
WT
yak1Δ
0% Glc (h)
0
0.5
1
0
0.5
1
IB
Hsf1
RT-PCR
BTN2
HSP26
CTT1
18s rRNA
0% Glc (h) 0 0.5 1
Relative mRNA level
WT
yak1Δ
BTN2
HSP26
CTT1
B
WT
yak1Δ
snf1Δ
yak1Δsnf1Δ
0% Glc (h)
BTN2
HSP26
CTT1
18S rRNA
0% Glc (h) 0 1
Relative mRNA level
snf1Δ
yak1Δsnf1Δ
C
39 °C (min)
0
15
18S rRNA
39 °C (min) 0 15

Figure 4.2 Hsf1 and Msn2/4 are controlled by Yak1 in response to acute glucose depletion. (A) Yak1 is involved in phosphorylation and activation of Hsf1 in response to acute glucose depletion. BY4741 (WT) and *yak1Δ* cells were grown in YPD medium containing 2% glucose until early exponential phase and then shifted to medium without glucose for the indicated times. Phosphorylation of Hsf1 was detected by immunoblotting and mRNA expression levels were detected by RT-PCR. In the right panel, transcript levels of *BTN2*, *HSP26*, and *CTT1* from three independent experiments were normalized to *18s rRNA* levels, and the relative mRNA levels were indicated using the transcript levels of unstressed wild type as a control. (B) Yak1, but not Snf1 is responsible for activation of Hsf1 and Msn2/4 in response to acute glucose depletion. BY4741 (WT) and its isogenic *yak1Δ*, *snf1Δ*, and *yak1Δ snf1Δ* strains were starved for glucose for 1 h. Levels of mRNA expression were detected by RT-PCR. (C) Yak1 is involved in activation of Msn2/4, but not Hsf1 by heat shock. WT and *yak1Δ* cells grown in YPD medium at 30^{o}C were heat shocked at 39^{o}C for 15 min, and the mRNA expression levels were analyzed by RT-PCR.

again reduced by additional deletion of *YAK1*, demonstrating the independent and opposite regulatory roles for Snf1 and Yak1 on Msn2/4 activity.

Next, the effect of Yak1 on activation of Hsf1 and Msn2/4 upon heat shock was examined (Fig. 4.2C). After 15 min of heat shock at 39 ^{o}C, the induction fold of *BTN2* mRNA was almost the same in wild type and *yak1Δ*, showing 14.1 and 13.1 fold induction, respectively, and induction of *HSP26* was only marginally reduced in *yak1Δ* (from 21.0 fold to 16.7 fold). However, *CTT1* induction was clearly reduced in *yak1Δ* (8.6 fold) than wild type (19.0 fold). These results suggest that Yak1 might be also involved in activation of Msn2/4 upon heat shock without playing a significant role in heat shock activation of Hsf1.

4.5 Yak1-dependent activation of Hsf1 and Msn2/4 is regulated by PKA but not by TORC1 pathway

PKA has been suggested to repress expression of small heat shock genes through indirect regulation of Hsf1 phosphorylation (Ferguson et al., 2005). Since Yak1 is under the negative control of PKA, Yak1 could possibly serve as one of the missing linkages mediating PKA-dependent repression of Hsf1. In order to test whether the Yak1-

dependent activation of Hsf1 and Msn2/4 is regulated by PKA, mRNA expression levels of the Hsf1- and Msn2/4-regulated genes were examined under the conditions of low PKA activity, which could be achieved by reducing cellular cAMP levels by overexpressing phosphodiesterase encoded by *PDE2* (Thevelein & de Winde, 1999). As expected, Pde2 overexpression led to the activation of Msn2/4, resulting in around 6-fold induction of *CTT1* and *HSP26* expression at early exponential phase (Fig. 4.3A). In addition, consistent with the PKA-dependent repression of Hsf1, *BTN2* mRNA level was also elevated by 1.7 fold in cells overexpressing Pde2, although the induction fold was lower than those of Msn2/4 targets. Pde2-dependent induction of *BTN2*, *HSP26*, and *CTT1* was all diminished in *yak1Δ*, demonstrating that Yak1 is involved in activation of Hsf1 and Msn2/4 upon inactivation of PKA (Fig. 4.3A).

Accumulating evidences suggest multiple ways of crosstalk between two nutrient-sensing kinases, PKA and TOR (Chen & Powers, 2006). Previously, TOR has been shown to regulate Yak1 activity via PKA in controlling ribosomal protein gene expression (Martin et al., 2004). In addition, nuclear localization of Yak1 has been shown to be induced by rapamycin treatment (Schmelzle et al., 2004) as well as by glucose depletion (Moriya et al., 2001). Therefore, it was investigated

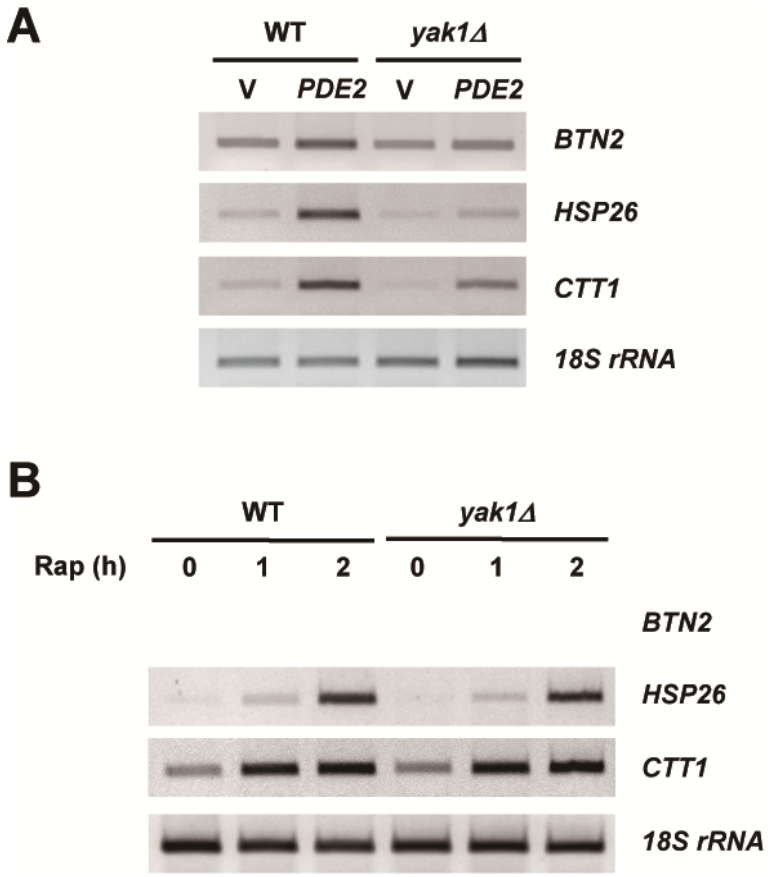

A
WT
yak1Δ
V
PDE2
V
PDE2
BTN2
HSP26
CTT1
18S rRNA
B
WT
yak1Δ
Rap (h)
0
1
2
0
1
2
BTN2
HSP26
CTT1
18S rRNA

Figure 4.3 PKA, but not TORC1 pathway controls Yak1-dependent activation of Hsf1 and Msn2/4. (A) Yak1 is required for activation of Hsf1 and Msn2/4 upon inactivation of PKA by overexpressing Pde2. WT and *yak1Δ* strains harboring empty vector (V) or a plasmid overexpressing *PDE2* (YEplac*PDE2*) were grown in SC-Ura medium until early log phase, and total RNA prepared from each sample was subjected to RT-PCR analysis. (B) Yak1 is not responsible for expression of Hsf1- and Msn2/4-dependent genes upon TOR inactivation. WT and *yak1Δ* cells were grown in YPD medium until early log phase and then treated with rapamycin (200 ng/ml) for the indicated times. Levels of mRNA expression were detected by RT-PCR.

whether TOR could regulate Yak1-dependent activation of Hsf1 or Msn2/4. In agreement with the TOR-dependent inhibition of Msn2/4 (Santhanam et al., 2004), inhibition of TOR by rapamycin treatment induced Msn2/4-regulated genes such as *HSP26* and *CTT1* (Fig. 4.3B). However, deletion of *YAK1* showed no defect in rapamycin induction of *HSP26* and *CTT1*, excluding the possibility that Yak1 might participate in TOR signaling pathway to regulate Msn2/4. On the other hand, *BTN2* mRNA levels were rather decreased by rapamycin treatment both in wild type and *yak1Δ* to the similar levels, indicating that Hsf1 is not the target of TOR pathway.

4.6 DNA binding activity of Hsf1 is increased by Yak1-dependent phosphorylation *in vitro*

Next, the Yak1- and Snf1-dependent phosphorylation sites of Hsf1 *in vitro* were compared. *In vitro* kinase assays using GST-Yak1 and GST-Snf1 kinases purified from *S. cerevisiae* showed differential specificity of these kinases toward various truncated Hsf1 (Fig. 4.4A). Hsf1 (1-180) was phosphorylated by Yak1, but not by Snf1. On the other hand, Hsf1 (147-583) was phosphorylated by Snf1, but not by Yak1. Although

more precise mapping of the phosphorylation sites is necessary, these preliminary experiments suggest that Yak1 and Snf1 regulate Hsf1 through phosphorylating different sites.

To eliminate the possibility that Hsf1 might be phosphorylated by unknown kinases co-purified with Yak1 in yeast, GST-Yak1 and a catalytically inactive GST-Yak1^{K398R} were expressed and purified in *E. coli*. Wild type, but not the mutant GST-Yak1 phosphorylated full-length or truncated versions of Hsf1 *in vitro*, confirming the Hsf1 kinase activity of Yak1 (Fig. 4.4B and C). The smaller phosphorylated bands of Hsf1 (1-883) and Hsf1 (1-583) were derived from protein degradation during their purification in *E. coli* (Fig. 4.4B). Yak1 is a member of evolutionally conserved DYRK dual-specificity protein kinases which have both tyrosine and serine/threonine kinase activity. It has been shown that DYRK protein kinases including Yak1 autophosphorylate on tyrosine and serine/threonine residues, but they do not appear to phosphorylate tyrosine residues of exogenous substrates (Himpel *et al.*, 2000, Kassis *et al.*, 2000). Accordingly, immunoblotting analysis with anti-phosphotyrosine antibody revealed the presence of phosphotyrosine in GST-Yak1, but not in GST-Hsf1 phosphorylated by Yak1 *in vitro* (data not shown).

Although Hsf1 trimer can bind to DNA under normal conditions, it has been shown that Hsf1 DNA binding affinity is induced upon

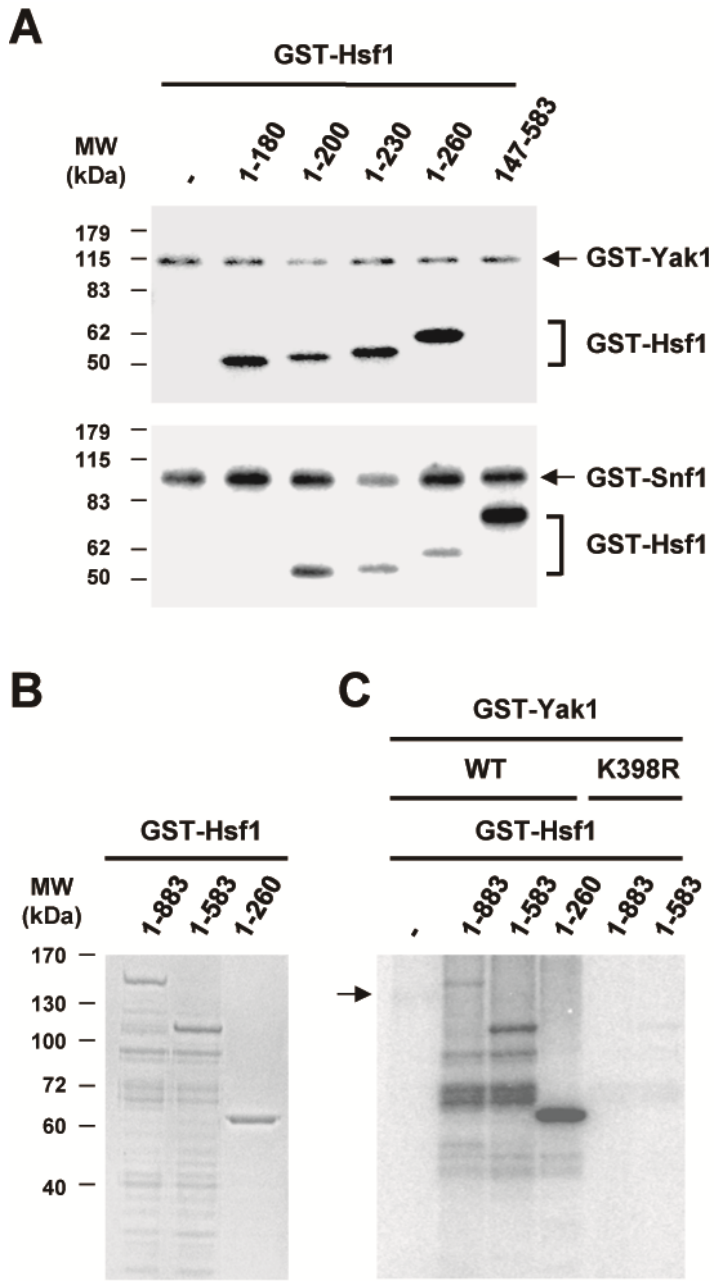

A
GST-Hsf1
MW
(kDa)
-
1-180
1-200
1-230
1-260
147-583
179
115
83
62
50
GST-Yak1
GST-Hsf1
179
115
83
62
50
GST-Snf1
GST-Hsf1
B
GST-Hsf1
MW
(kDa)
1-883
1-583
1-260
170
130
100
72
60
40
C
GST-Yak1
WT
K398R
GST-Hsf1
-
1-883
1-583
1-260
1-883
1-583

Figure 4.4 Hsf1 is phosphorylated *in vitro* by Yak1 purified from *S. cerevisiae* or *E. coli*. (A) Phosphorylation of GST-Hsf1 *in vitro* by Yak1 and Snf1 purified from *S. cerevisiae*. GST-Yak1 and SGT-Snf1 kinases were purified from *S. cerevisiae* and *in vitro* kinase assays were performed using various truncated derivatives of Hsf1 proteins, which had been expressed and purified in *E. coli* as GST-fusion proteins. Autophosphorylation of GST-Yak1 and SGT-Snf1 kinases, and phosphorylation of Hsf1 substrates were detected by SDS-PAGE followed by autoradiography. (B) GST-Hsf1 proteins used for kinase assays. Full length (1-833) or C-terminal truncated derivatives of GST-Hsf1 proteins were purified from *E. coli* and stained with Coommassie brilliant blue. (C) Phosphorylation Hsf1 *in vitro* by GST-Yak1 purified from *E. coli*. GST-Yak1 wild type and K398R mutant proteins were purified from *E. coli* and kinase assays were performed using various Hsf1 proteins shown in panel B as substrates. The autophosphorylated GST-Yak1 band is indicated by an arrow.

activation by heat shock or glucose starvation (Hahn et al., 2004, Hahn & Thiele, 2004). Therefore, it was asked whether Yak1-dependent phosphorylation could activate Hsf1 through enhancing DNA binding activity. Hsf1-regulated genes contain various types of HSEs in their promoters and the HSE architecture has been shown to be partly responsible for determining the DNA binding affinity of Hsf1 and also for differential regulation by phosphorylated Hsf1 (Hashikawa & Sakurai, 2004, Hashikawa *et al.*, 2006). Therefore, Hsf1 binding activity to three DNA probes containing different types of HSE was examined. As shown in Fig. 4.5A, HSE 3P and 5P contain three and five perfect inverted repeats of the nGAAn HSE unit, respectively, and HSE gap type contains three HSE units with a gap between the second and the third HSE. The Hsf1 target genes *BTN2*, *HSP26*, and *CUP1*, which it was used to examine transcriptional regulation contain 4P, 3P, and gap type HSE, respectively in their promoters (Hashikawa et al., 2006). GST-Hsf1 purified from *E. coli* was phosphorylated by GST-Yak1 or incubated with GST-Yak1^{K398R} as a control, and the DNA binding activity of GST-Hsf1 was detected by electrophoretic mobility shift assay using various concentrations of GST-Hsf1 (Fig. 4.5A). The fraction of Hsf1-bound DNA was plotted as a function of GST-Hsf1 concentration and the equilibrium dissociation constant (K_d) of GST-Hsf1 to each HSE was estimated from the GST-Hsf1 concentration

required for half-maximal binding. Unphosphorylated GST-Hsf1, which had been incubated with GST-Yak1^{K398R} bound to HSE 5P with the highest affinity (K_d = 32.8 nM) as expected. Unphosphorylated GST-Hsf1 bound to HSE 3P and gap type with much lower affinity showing K_d of 1.0 and 1.6 μM, respectively. GST-Hsf1 that had been phosphorylated by Yak1 showed increase in DNA binding activity to all three types of HSE probes, exhibiting decrease in K_d by 2.3 fold for gap type HSE, 2.1 fold for HSE 3P, and 1.6 fold for HSE 5P. Therefore, Yak1-dependent phosphorylation of Hsf1 might induce conformational change of Hsf1, resulting in the increase in DNA binding activity even to HSE 5P with high affinity. However, the effect of Hsf1 phosphorylation seems more prominent for atypical or weak HSEs in terms of fold change in K_d.

In order to test whether Yak1-dependent activation of Hsf1 can lead to HSE type-specific regulation *in vivo*, HSE reporter plasmid where 6P, 3P or gap type HSE is inserted upstream of the *CYC1* promoter fused to the *lacZ* gene (HSE-*CYC1-lacZ*) was used (Hashikawa et al., 2006). Wild type and *yak1Δ* carrying the reporter plasmids were grown to mid-exponential phase (OD_{600} of 3) and mRNA expression levels of the *lacZ* reporter were examined by RT-PCR (Fig. 4.5B). In agreement with the higher binding affinity of Hsf1, HSE 6P-

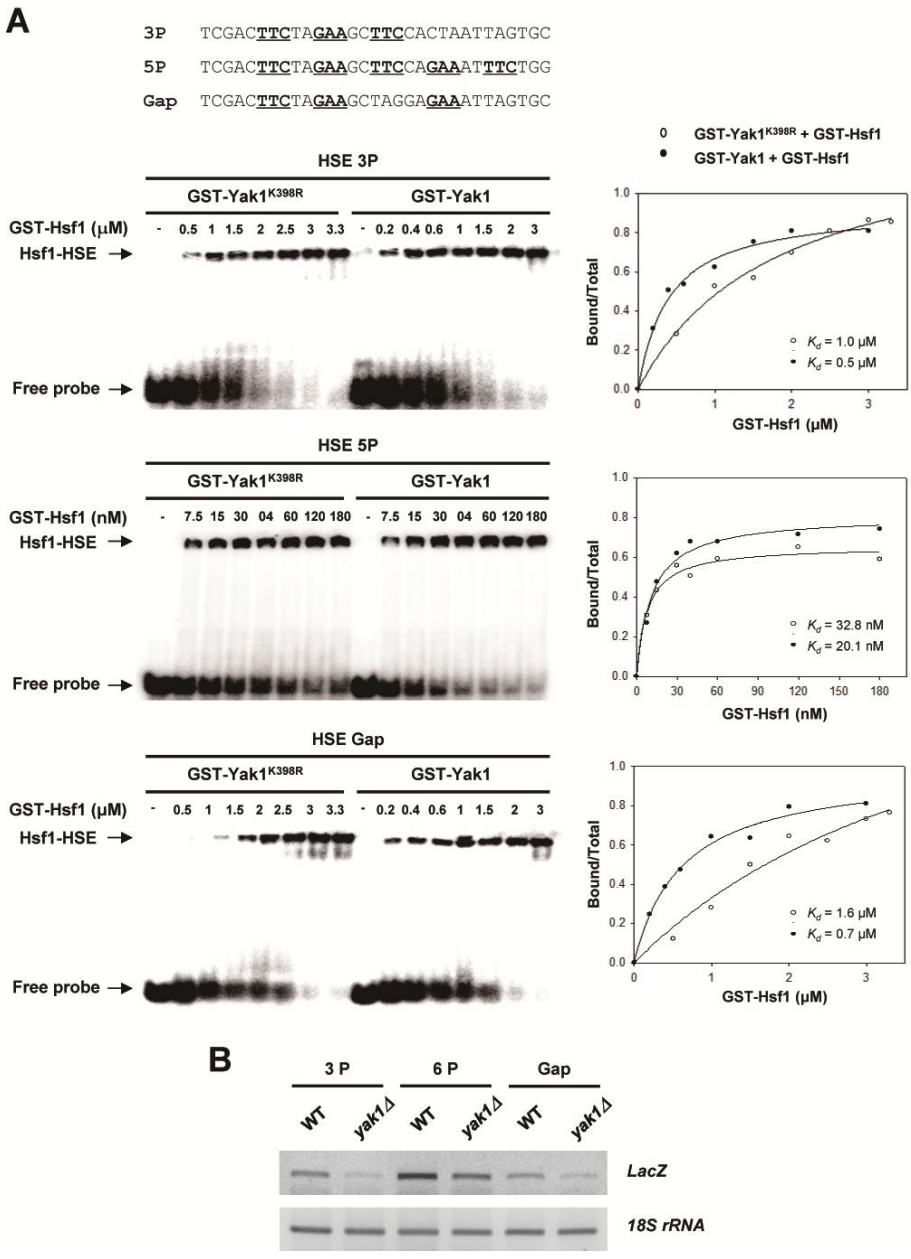

A
3P TCGACTTCTAGAAGCTTCCACTAATTAGTGC
5P TCGACTTCTAGAAGCTTCCAGAAATTTCTGG
Gap TCGACTTCTAGAAGCTAGGAGAAATTAGTGC
HSE 3P
GST-Yak1K398R
GST-Yak1
GST-Hsf1 (μM)
- 0.5 1 1.5 2 2.5 3 3.3 - 0.2 0.4 0.6 1 1.5 2 3
Hsf1-HSE
Free probe
0 GST-Yak1K398R + GST-Hsf1
GST-Yak1 + GST-Hsf1
Bound/Total
Kd = 1.0 μM
Kd = 0.5 μM
GST-Hsf1 (μM)
HSE 5P
GST-Hsf1 (nM)
- 7.5 15 30 04 60 120 180 - 7.5 15 30 04 60 120 180
Kd = 32.8 nM
Kd = 20.1 nM
GST-Hsf1 (nM)
HSE Gap
Kd = 1.6 μM
Kd = 0.7 μM
B
3 P
6 P
Gap
WT
yak1Δ
LacZ
18S rRNA

Figure 4.5 Yak1-dependent phosphorylation of Hsf1 enhances DNA binding activity of Hsf1 *in vitro*. (A) Increase in Hsf1 DNA-binding affinity by Yak1-dependent phosphorylation. Three different types of HSE (3P, 5P, and Gap type) with the indicated nucleotide sequences (repeating GAA or TTC units are underlined) were used as probes for electrophoresis mobility shift assay. GST-Hsf1 purified from *E. coli* was phosphorylated by wild type GST-Yak1 or incubated with the same amount of mutant GST-Yak1^{K398R} as a control for 90 min. The reaction mixtures containing the indicated amount of GST-Hsf1 were incubated with ^{32}P-end-labeled DNA fragments containing 3P, 5P, or gap type HSE at room temperature for 15 min. The samples were subjected to 6%-PAGE and phosphorimager analysis to monitor Hsf1 binding to HSE. The values of radioactivity in bound and free DNA were used to plot the fraction of bound DNA as a function of GST-Hsf1 concentration. The equilibrium dissociation constant (K_d) was estimated from the GST-Hsf1 protein concentration required for half-maximal binding to each probe DNA. (B) Role of Yak1 in transcription of HSE-*CYC1-LacZ* reporters. WT and *yak1*Δ cells carrying HSE 3P-*CYC1-lacZ*, HSE 6P-*CYC1-lacZ*, and HSE Gap-*CYC1-lacZ* reporters were grown in SC-Ura medium until OD_{600} of 3, and the *lacZ* mRNA expression levels were detected by RT-PCR. *18S rRNA* was used as a

control. Relative *lacZ* mRNA levels from three independent experiments normalized to 18s rRNA levels were shown in the right panel.

CYC1-lacZ showed higher expression levels than the reporters containing 3P or Gap type HSE both in wild type and *yak1Δ*. In addition, the *lacZ* expression derived from all three HSE types were reduced in *yak1Δ* compared with wild type, clearly demonstrating that Yak1 is involved in activation of Hsf1. However, significant difference in Yak1-dependent expression patterns among the three HSE types could not be detected. The *lacZ* expression was reduced in *yak1Δ* by 1.8 fold for both HSE 6P and 3P reporters and 2.3 fold for gap type HSE reporter. Therefore, HSE architecture might not be a critical factor for determining Yak1-dependent regulation of Hsf1 target genes. The fact that expression of *BTN2* containing HSE 4P with relatively high affinity is affected by *YAK1* deletion to the comparable extent to that of *CUP1* containing gap type HSE also supports the view that Yak1 might regulate Hsf1 target genes with various types of HSE (Fig. 4.1B).

4.7 Yak1 phosphorylates Msn2 *in vitro*

In addition to the regulation of Hsf1, Yak1-dependent activation of Msn2/4 was reproducibly observed. The role for Yak1 in activation of Msn2/4 could be achieved by either direct or indirect pathways. The simplest hypothesis is the direct phosphorylation of Msn2/4 by Yak1 as

demonstrated for the regulation of Hsf1. In order to test this possibility, Full length and N- and C- terminal domains of Msn2 from *E. coli* as GST-fusion proteins and performed *in vitro* kinase assay using GST-Yak1 were purified (Fig. 4.6 A and B). Yak1 phosphorylated the full length and C-terminal domain (401-704) of Msn2, but not the N-terminal domain (1-400). Note that the phosphorylated full length GST-Msn2 band is located slightly below the autophosphorylated band of GST-Yak1 kinase. Phosphorylation of the full length Msn2 by kinase assay with reduced amount of Yak1 kinase was confirmed (data not shown). These results support the notion that Yak1 may activate Msn2 by direct phosphorylation.

In order to understand the mechanism for activation of Msn2 by Yak1-dependent phosphorylated, it was first tested whether Yak1 could alter DNA binding activity of Msn2 as shown for Hsf1. For yet unknown reason, DNA binding activity of the full length Msn2 in our *in vitro* DNA binding assay conditions could not be detected. However, the GST-Msn2 (401-704) which contains zinc finger DNA binding domain bound to *CTT1* promoter containing two STREs. Therefore, DNA binding activity of GST-Msn2 (401-704) after phosphorylation by Yak1 was examined (Fig. 4.6C). Unlike Hsf1, phosphorylated GST-Msn2 (401-704) showed almost the same binding activity to *CTT1* promoter compared with GST-Msn2 (401-704) incubated with GST-Yak1^{K398R}.

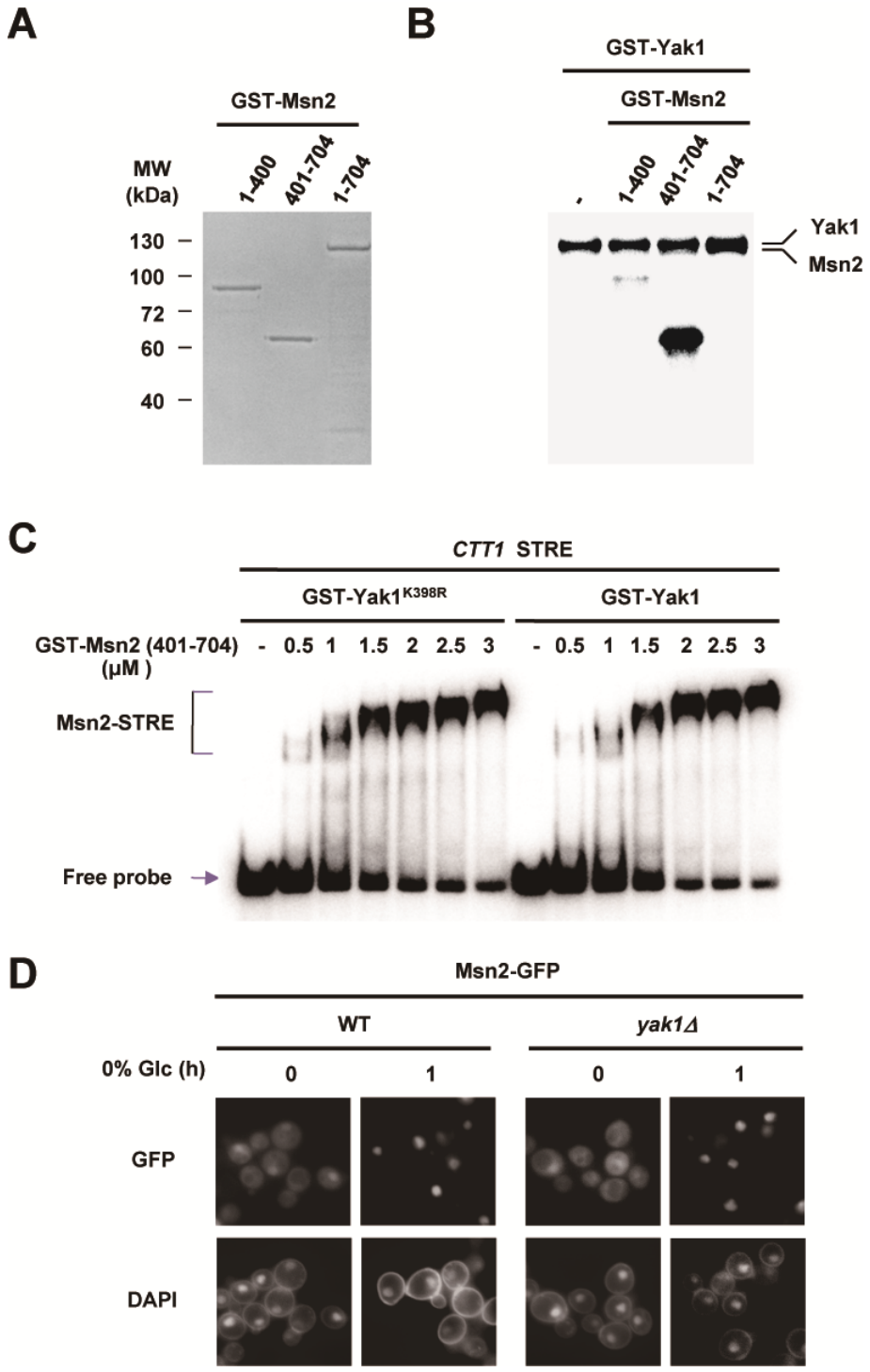

A
GST-Msn2
MW
(kDa)
1-400
401-704
1-704
130
100
72
60
40
B
GST-Yak1
GST-Msn2
-
1-400
401-704
1-704
Yak1
Msn2
C
CTT1 STRE
GST-Yak1K398R
GST-Yak1
GST-Msn2 (401-704)
(μM)
- 0.5 1 1.5 2 2.5 3
- 0.5 1 1.5 2 2.5 3
Msn2-STRE
Free probe
D
Msn2-GFP
WT
yak1Δ
0% Glc (h)
0
1
0
1
GFP
DAPI

Figure 4.6 Yak1 phosphorylates Msn2 *in vitro*. (A) GST-Msn2 proteins used for kinase assays. Coommassie-stained image of GST-Msn2 full length (1-704) or truncated derivatives purified from *E. coli*. (B) Phosphorylation of Msn2 C-terminal domain by Yak1 *in vitro*. The GST-Msn2 proteins shown in panel A were phosphorylated by GST-Yak1 purified from *E. coli*. The locations of autophosphorylated GST-Yak1 and phosphorylated full length GST-Msn2 are indicated. (C) Yak1-dependent phosphorylation does not affect DNA binding activity of Msn2 (401-704). GST-Msn2 (401-704) was phosphorylated by GST-Yak1 or incubated with the same amount of GST-Yak1^{K398R} as a control for 90 min. The reaction mixtures containing the indicated amount of GST-Msn2 (401-704) were incubated with ^{32}P-end-labeled *CTT1* promoter (-400 to -300) containing two STREs at room temperature for 15 min. The samples were subjected to 6%-PAGE and phosphorimager analysis to monitor GST-Msn2 (401-704) binding to STRE. (D) Yak1 does not affect Msn2 nuclear localization upon acute glucose depletion. BY4741 (WT) or *yak1*Δ cells expressing Msn2-GFP were grown in selective SC medium containing 2% glucose until early log phase and then shifted to medium without glucose for 1 h. Localization of Msn2-GFP and DAPI-stained nucleus were detected by fluorescence microscopy.

Although it cannot be ruled out the possibility that Yak1 might regulate Msn2 DNA binding activity in the context of full length Msn2 protein, phosphorylation of C-terminal domain of Msn2 seems not affect its DNA binding activity.

The C-terminal half of Msn2, phosphorylated by Yak1, also contains nuclear localization signal (NLS) which is under the control of PKA- and Snf1-dependent phosphorylation (De Wever et al., 2005, Gorner et al., 1998, Gorner et al., 2002). Therefore, it was asked whether Yak1 could affect Msn2 nuclear localization upon glucose depletion. However, in agreement with the previously observation showing that Yak1 does not affect oscillatory nucleocytoplasmic shuttling of Msn2 (Jacquet *et al.*, 2003), deletion of Yak1 did not affect nuclear localization of Msn2-GFP upon acute glucose depletion (Fig. 4.6D). Both in wild type and *yak1Δ*, Msn2-GFP was localized throughout the cell in the presence of 2% glucose and translocated to the nucleus upon glucose depletion. Taken together, these results suggest that Yak1 may activate Msn2 after the event of nuclear localization and DNA binding.

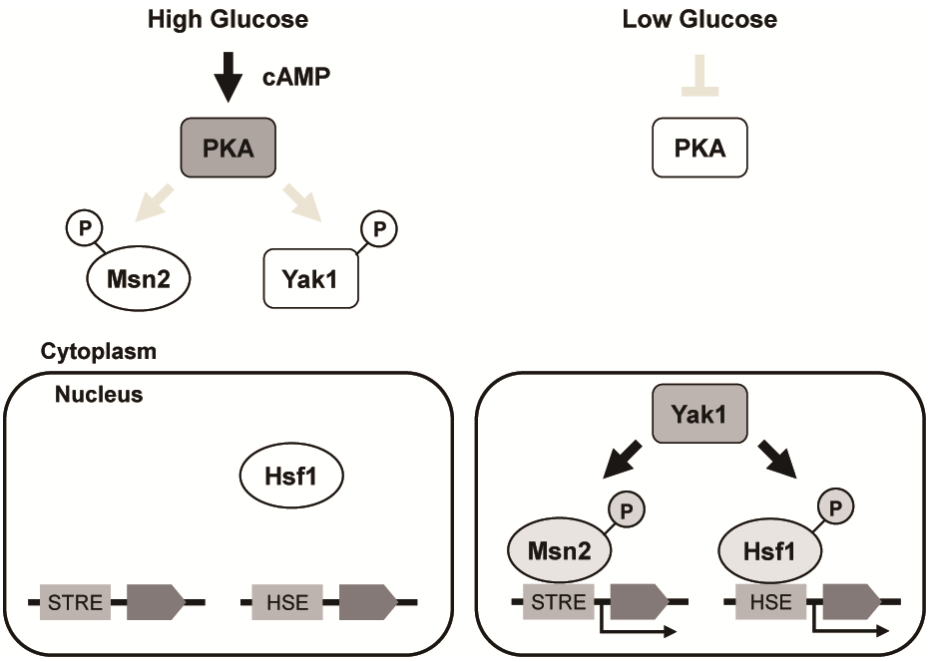


Figure 4.7 Model of Yak1-dependent regulation of Hsf1 and Msn2. Under high glucose conditions, PKA inhibits nuclear localization of Msn2 by direct phosphorylation. PKA also phosphorylates and inhibits Yak1 by yet unidentified mechanism. Inhibition of the PKA activity by glucose limitation allows translocation of Msn2 and Yak1 to the nucleus. Yak1 then phosphorylates and activates Msn2 and Hsf1. Phosphorylation of Hsf1 increase its DNA binding activity, but the mechanism for Yak1-dependent Msn2 activation is still unknown.

4.8 Conclusions

In this chapter, Yak1 kinase has been shown to activate Hsf1 by direct phosphorylation upon inactivation of PKA. Moremore, Msn2 has been shown to be activated by Yak1 via direct phosphorylation. Therefore, these data suggest an important role for Yak1 in mediating PKA-dependent inhibition of stress responses. This study includes biologically important and novel findings, which would contribute to understanding the regulatory networks balancing cell growth and stress adaptation in response to nutrient availability.

Chapter 5.

Rim15-dependent activation of Hsf1 and Msn2/4 transcription factors by direct phosphorylation

5.1 Introduction

In *Saccharomyces cerevisiae*, nutrient-sensing kinases PKA (protein kinase A) and TORC1 (target of rapamycin complex 1) integrate the nutrient signals to regulate cell proliferation by stimulating ribosome biogenesis and translation while inhibiting stress responses and autophagy (Yorimitsu *et al.*, 2007, Wei *et al.*, 2008, Roosen *et al.*, 2005). In line with their functional similarity, PKA and TORC1 signaling pathways share several common mediators including Yak1, a member of Ser/Thr kinases known as dual-specificity Tyr phosphorylation-regulated kinases (DYRKs) (Martin et al., 2004, Schmelzle et al., 2004), and Rim15, a distinct member of the PAS family (Pedruzzi et al., 2003, Reinders et al., 1998).

Yak1 and Rim15 are negatively regulated by PKA and TORC1 by different mechanisms. PKA-dependent phosphorylation inhibits the nuclear localization of Yak1 (Lee *et al.*, 2011), whereas PKA inhibits the kinase activity of Rim15 (Reinders et al., 1998). Inhibition of TORC1 leads to the nuclear localization of both Yak1 and Rim15. TORC1-dependent cytoplasmic retention of Rim15 is mediated by Sch9, which phosphorylates Rim15, providing a binding site of Bmh1 and Bmh2, yeast 14-3-3 proteins (Pedruzzi et al., 2003, Wanke et al., 2005). However, the mechanism for the TORC1-dependent regulation

of Yak1 is not well understood (Schmelzle et al., 2004). Bmh1/2 also binds to Yak1 in the presence of glucose, but Bmh1/2 repress the kinase activity of Yak1 rather than regulating its cellular localization (Lee et al., 2011).

Upon nutrient starvation, Yak1 and Rim15 exert their effects on growth inhibition and initiation of the quiescence (G_0) program in part by regulation of gene expression. Rim15 up-regulates the expression of a variety of genes whose expression depends on zinc finger transcription factors Msn2, Msn4, and Gis1 (Cameroni *et al.*, 2004). Msn2/4 activate gene expression by binding to stress response element (STRE, AGGGG) in response to diverse environmental stresses including heat shock, oxidative stress, and nutrient starvation (Gorner et al., 1998, Martinez-Pastor et al., 1996). Gis1 activates transcription through post diauxic shift (PDS, TT/AAGGGAT) element upon glucose depletion at the diauxic shift (Pedruzzi et al., 2003). Msn2/4 and Gis1 have functional overlaps, cooperatively regulating many common targets (Cameroni et al., 2004). However, it is not known how Rim15 regulates the Msn2/4- and Gis1-dependent transcriptional activation. In addition to the transcriptional activation, Rim15 has been shown to regulate gene expression by posttranscriptional mRNA stabilization. Igo1 and Igo2, which are phosphorylated by Rim15, antagonize decapping of specific nutrient-

regulated mRNAs, thus preventing their 5' to 3' degradation (Luo *et al.*, 2011, Talarek *et al.*, 2010).

Previously in chapter4, it was shown that Yak1 activates Msn2 by direct phosphorylation (Lee *et al.*, 2008). Although nuclear localization of Msn2/4 is regulated by PKA-dependent phosphorylation (De Wever et al., 2005), Yak1 might be necessary for the activation of Msn2/4 after the event of DNA binding. In addition, it was also shown that Yak1 phosphorylates and activates heat shock transcription factor Hsf1 upon glucose depletion through enhancing its DNA binding affinity (Lee et al., 2008).

In this chapter, the role for Rim15 in the expression of Hsf1 target genes was newly demonstrated. It was showed that Rim15 can regulate the expression of Hsf1 target genes by both phosphorylation-dependent activation of Hsf1 and Igo1/2-dependent stabilization of the corresponding transcripts. Rim15 also phosphorylates Msn2, but not Gis1, *in vitro*, suggesting that Yak1 and Rim15 converge on the same targets, Hsf1 and Msn2, to cope with nutrient starvation.

5.2 Both Rim15 and Yak1 are involved in the transcriptional activation of Hsf1 and Msn2/4 target genes

Previously in chapter4, it was demonstrated that Yak1 activates Hsf1 and Msn2/4 by direct phosphorylation. Based on a functional similarity between Yak1 and Rim15, it was asked whether Rim15 is also involved in the regulation of Hsf1. First, the effect of *RIM15* deletion on the expression of Hsf1 target genes under the conditions of acute glucose depletion was examined (Fig. 5.1A). Glucose starvation-dependent induction of *BTN2*, an Hsf1 target gene, was reduced in *yak1Δ* than wild type as expected. Deletion of *RIM15* also reduced the induction of *BTN2*, indicating that both Yak1 and Rim15 are involved in the activation of Hsf1 target genes upon glucose starvation. *BTN2* induction was further reduced in *yak1Δrim15Δ*, suggesting a combinatorial effect of Rim15 and Yak1 in *BTN2* expression. *HSP26*, which are regulated by both Hsf1 and Msn2/4, showed Yak1- and Rim15-dependent induction patterns similar to those of *BTN2* (Fig.5.1A). In agreement with previous result in chapter4 showing that Yak1 is involved in the activation of Msn2/4, deletion of *YAK1* reduced the glucose starvation-dependent induction of *CTT1*, an Msn2/4 target. Although Rim15 is also known to activate Msn2/4 target genes, *CTT1* induction was not much affected by the lack of Rim15 under our glucose depletions conditions (Fig.5.1A).

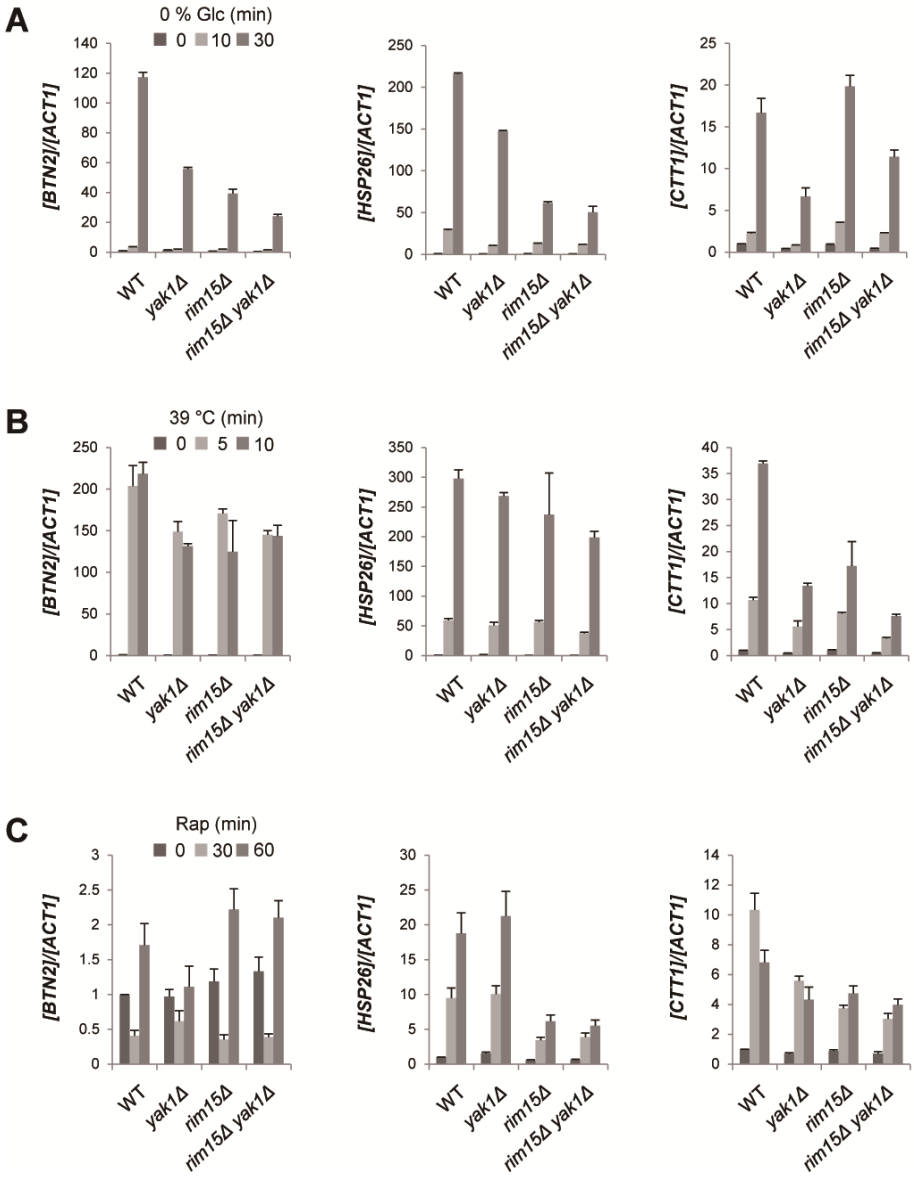

A
0 % Glc (min)
0 10 30
[BTN2]/[ACT1]
[HSP26]/[ACT1]
[CTT1]/[ACT1]
WT
yak1Δ
rim15Δ
rim15Δ yak1Δ
B
39 °C (min)
0 5 10
C
Rap (min)
0 30 60

Figure 5.1 Roles of Yak1 and Rim15 in the expression of Hsf1 and Msn2/4 target genes under stress conditions. Wild type (WT), *yak1Δ*, *rim15Δ*, and *rim15Δyak1Δ* cells were grown in YPD medium containing 2% glucose until early exponential phase and then shifted to YP medium lacking glucose (A), heat shocked at 39^{o}C (B), or treated with 200 ng/ml rapamycin for the indicated times. The mRNA levels of *BTN2*, *HSP26*, and *CTT1* were quantified by qRT-PCR and normalized to *ACT1* mRNA. The experiments were done in triplicates.

Previously reported In chapter4, it was shown that Yak1 contributes to the activation of Msn2 upon heat shock without playing a critical role in heat shock activation of Hsf1. Accordingly, deletion of *YAK1* clearly reduced the induction of *CTT1* upon heat shock, but exerted marginal effects on the induction of *BTN2* and *HSP26* (Fig. 5.1B). The weak effect of Yak1 and Rim15 on the heat shock induction of Hsf1 target genes might be caused by a dominant role of PKA-independent pathway for the heat shock activation of Hsf1. *CTT1* induction was also reduced in *rim15Δ*, indicating that Rim15 is involved in heat shock induction of Msn2/4 target genes. The double deletion strain *rim15Δyak1Δ* showed greater defects in *CTT1* and *HSP26* induction than each single deletion mutant, suggesting that Yak1 and Rim15 act in parallel to activate Msn2/4 target genes upon heat shock.

Next, the role for Rim15 and Yak1 in gene expression upon inhibition of TORC1 by rapamycin was investigated. As previously reported, *BTN2* mRNA level was not significantly increased by rapamycin treatment and rather slightly reduced after 30 min of TORC1 inactivation (Fig. 5.1C). Deletion of neither *YAK1* nor *RIM15* exerted significant effect on *BTN2* expression upon rapamycin treatment. On the other hand, deletion of *RIM15* reduced the rapamycin-dependent induction of *HSP26* and *CTT1*, confirming the

role for Rim15 in activation of Msn2/4 upon TORC1 inhibition. *CTT1* induction was also reduced by the lack of *YAK1*.

Taken together, it was newly discovered that Rim15 is involved in the activation of Hsf1 targets upon glucose starvation. Yak1 and Rim15 might act downstream of PKA to commonly regulate Hsf1 and Msn2/4 targets upon glucose starvation and heat shock, but their effects seem to be different depending on the promoter context of the targets. For TORC1-dependent regulation of Msn2/4, Rim15 seems to play a larger role than Yak1.

5.3 Hsf1 is phosphorylated by Rim15 *in vitro*

Next, it was investigated whether the Rim15-dependent activation of Hsf1 target genes is mediated by direct phosphorylation of Hsf1 by Rim15. To eliminate the possible co-purification of Yak1, which is known to phosphorylate Hsf1, GST-Rim15 in *rim15Δyak1Δ* strain was purified. Igo1, the known substrate of Rim15 (Talarek et al., 2010) was used as a positive control for *in vitro* kinase assay. GST-Igo1 and GST-Hsf1 were expressed and purified in *E. coli* (Fig. 5.2A). As shown in Fig 5.2B, GST-Rim15, but not a kinase dead mutant GST-Rim15$^{K823Y/C1176Y}$, could phosphorylate Hsf1 as well as Igo1 *in vitro*.

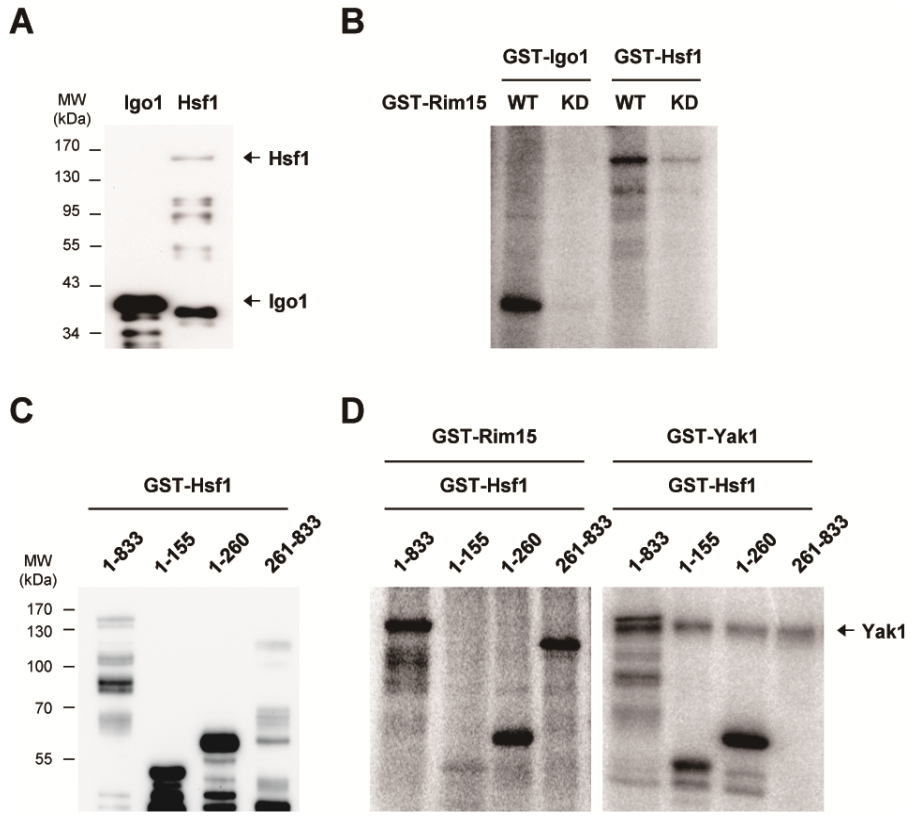

A
MW (kDa)
Igo1
Hsf1
170
130
95
55
43
34
Hsf1
Igo1
B
GST-Igo1
GST-Hsf1
GST-Rim15
WT
KD
WT
KD
C
GST-Hsf1
1-833
1-155
1-260
261-833
MW (kDa)
170
130
100
70
55
D
GST-Rim15
GST-Yak1
GST-Hsf1
GST-Hsf1
1-833
1-155
1-260
261-833
1-833
1-155
1-260
261-833
Yak1

Figure 5.2 Rim15 phosphorylates Hsf1 *in vitro*. (A) GST-Igo1 and GST-Hsf1 proteins used for kinase assays. GST-fused Igo1 and Hsf1 proteins were purified from *E. coli* and detected by immunoblotting using anti-GST antibody. (B) Phosphorylation of GST-Igo1 and GST-Hsf1 *in vitro* by Rim15. GST-Rim15 wild type (WT) and kinase dead K823Y/C1176Y (KD) mutant proteins were purified from *rim15Δyak1Δ* strain and *in vitro* kinase assays were performed using GST-Igo1 and GST-Hsf1 as substrates. Phosphorylated bands were detected by SDS-PAGE followed by autoradiography. (C) GST-Hsf1 full length (1-833) and truncation mutants used for kinase assays. Proteins were purified from *E.coli* and detected by immunoblotting using anti-GST antibody. (D) Phosphorylation of GST-Hsf1 *in vitro* by Yak1 and Rim15. GST-Yak1 and GST-Rim15 kinases were purified from *E.coli* and *S.cerevisiae*, respectively, and *in vitro* kinase assays were performed using various truncated forms of GST-Hsf1 proteins. Autophosphorylation band of GST-Yak1 was indicated.

Since Yak1 also phosphorylates Hsf1, the Yak1- and Rim15-dependent phosphorylation sites in Hsf1 by using various truncation mutants of Hsf1 were compared (Fig. 5.2C). Although GST-Hsf1 (1-260) was phosphorylated by both Rim15 and Yak1, GST-Hsf1 (1-155) was phosphorylated by Yak1, but not by Rim15 (Fig. 5.2D). On the other hand, only Rim15 could phosphorylate GST-Hsf1 (261-833), indicating that Rim15 and Yak1 might activate Hsf1 through phosphorylating different sites.

5.4 BTN2 mRNA is stabilized by Igo1/2 upon glucose starvation

Rim15 plays a dual role in gene regulation by transcriptional activation and protection of the transcripts from degradation via phosphorylating Igo1/2 (Talarek et al., 2010, Luo et al., 2011). It has been shown that rapamycin-dependent induction of the entire Rim15-regulated genes requires Igo1/2 (Talarek et al., 2010). Although it was suggested that Rim15-depedent phosphorylation and subsequent activation of Hsf1 might be responsible for the Rim15-dependent induction of *BTN2* upon glucose starvation, it cannot be ruled out the possibility that Igo1/2-dependent stabilization of *BTN2* transcript is also part of the Rim15-

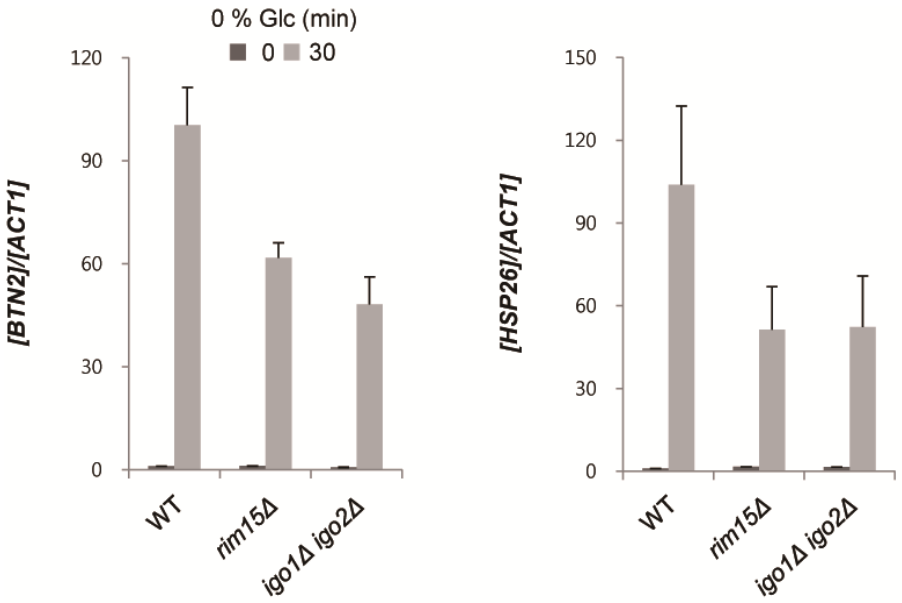


Figure 5.3 Igo1/2 are involved in the induction of *BTN2* mRNA upon glucose starvation. WT, *rim15Δ*, and *igo1Δigo2Δ* cells were grown in YPD medium until early exponential phase and then shifted to medium without glucose for 30 min. *BTN2* and *HSP26* mRNA levels were detected by qRT-PCR and normalized to *ACT1* mRNA.

dependent induction mechanism. To test this possibility, the effect of *IGO1* and *IGO2* deletion on the expression of *BTN2* upon glucose starvation was investigated. Previously, it has been shown that Igo1 and Igo2 are involved in the stabilization of *HSP26* mRNA following rapamycin treatment (Talarek et al., 2010). As shown in Fig 5.3, glucose starvation-dependent induction of *BTN2* as well as *HSP26* was reduced in both *rim15Δ* and *igo1Δigo2Δ* compared with WT, suggesting that Igo1/2-dependent stabilization of *BTN2* mRNA may also contributes to the induction of *BTN2* level upon glucose starvation.

5.5 Rim15 phosphorylates Msn2, but not Gis1, *in vitro*

Although Rim15 is known to be involved in the transcriptional activation of Msn2/4 and the Gis1 target genes (Cameroni et al., 2004), the activation mechanisms are largely unknown. Since direct phosphorylation of Hsf1 by Rim15 was detected, it was also examined whether Msn2 and Gis1 are direct substrates of Rim15. Interestingly, Rim15 could directly phosphorylate Msn2, but not Gis1, *in vitro* (Fig. 5.4A and B). Rim15 phosphorylated both N-terminal domain (1-400) and C-terminal domain (401-704) of Msn2 (Fig. 5.4C and D).

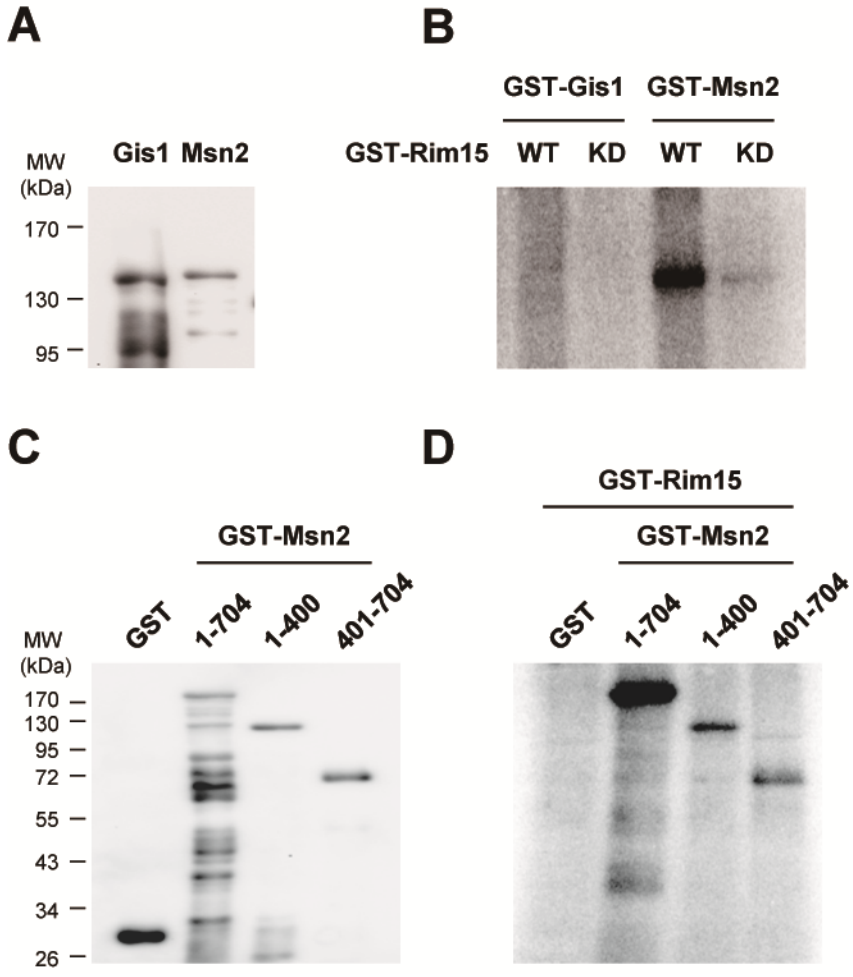

A
Gis1
Msn2
MW
(kDa)
170
130
95
B
GST-Gis1
GST-Msn2
GST-Rim15
WT
KD
WT
KD
C
GST-Msn2
GST
1-704
1-400
401-704
MW
(kDa)
170
130
95
72
55
43
34
26
D
GST-Rim15
GST-Msn2
GST
1-704
1-400
401-704

Figure 5.4 Rim15 phosphorylates Msn2, but not Gis1, *in vitro*. (A) GST-Gis1 and GST-Msn2 proteins used for kinase assays. GST-fused Gis1 and Msn2 proteins were purified from *E. coli* and detected by immunoblotting using anti-GST antibody. (B) The GST-Msn2 and GST-Gis1 proteins shown in panel A were phosphorylated by GST-Rim15 (WT) and GST-Rim15$^{K823Y/C1176Y}$ (KD) purified from *S.cerevisiae*. (C) GST-Msn2 derivatives used for kinase assays. GST-Msn2 full length (1-704) and its truncated derivatives were purified from *E. coli* and detected by immunoblotting using anti-GST antibody. (D) The GST-Msn2 and its truncated derivatives shown in panel C were phosphorylated by GST-Rim15 *in vitro*.

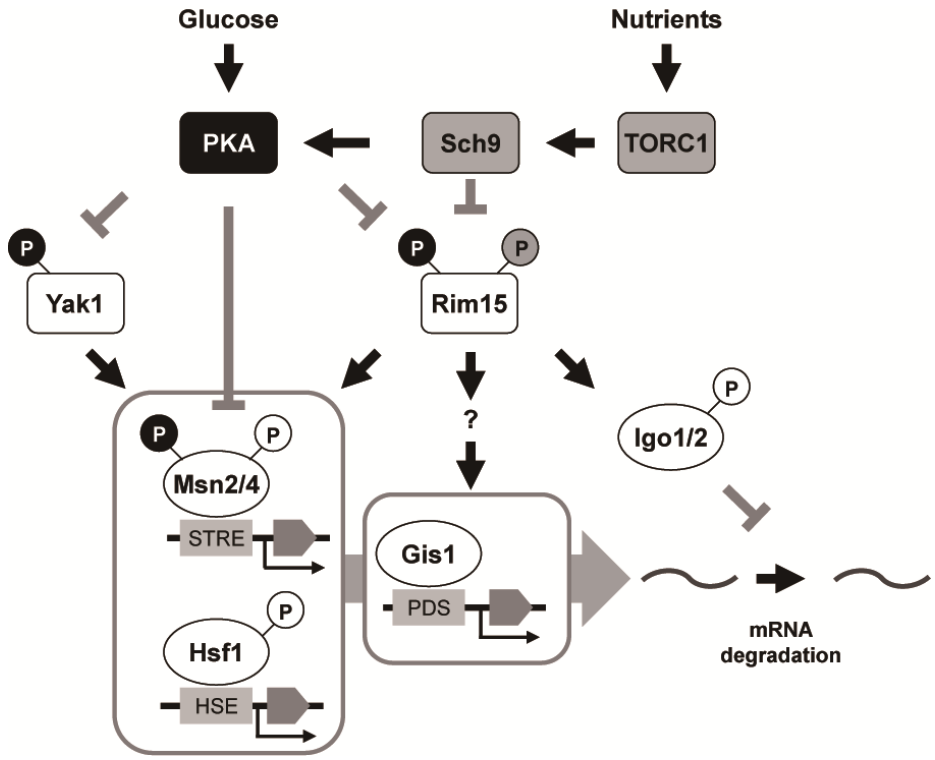

Glucose
Nutrients
PKA
Sch9
TORC1
P
Yak1
P
P
Rim15
?
P
Msn2/4
STRE
P
Hsf1
HSE
Gis1
PDS
P
Igo1/2
mRNA
degradation

Figure 5.5 A model for the Yak1- and Rim15-dependent regulation of Hsf1 and Msn2/4 target genes. Under high glucose conditions, PKA inhibits the nuclear localization of Yak1 and Msn2/4, and the kinase activity of Rim15 by direct phosphorylation (black circle). Sch9, a downstream kinase of TORC1, phosphorylates Rim15, thus inhibiting its nuclear localization (gray circle). TORC1 also controls some PKA targets though activation of PKA via Sch9. Upon nutrient starvation, active Yak1 and Rim15 in the nucleus activate Hsf1 and Msn2/4 by direct phosphorylation (white circle). Rim15 also activates the expression of Gis1 targets by yet unidentified mechanism. On the other hand, Rim15-dependent phosphorylation of Igo1/2 prevents degradation of the induced transcripts.

Previously in chapter 4, it has been shown that Yak1 can activate Msn2 by direct phosphorylation on the C-terminal domain (401-704), implying that Yak1 and Rim15 might regulate Msn2 through phosphorylating different sites.

5.6 Conclusions

In this chapter, Rim15 is shown to be involved in the activation of Hsf1 targets by both phosphorylation-dependent activation of Hsf1 and Igo1/2-dependent stabilization of the corresponding transcripts. Furthermore, it has been demonstrated that Rim15 also phosphorylates Msn2, but not Gis1, *in vitro*, suggesting different mechanisms for the activation of these transcription factors. Taken together with the previous observation that Yak1 kinase is involved in the activation of Hsf1 and Msn2/4 by direct phosphorylation, the study presented here suggests cooperative roles of Rim15 and Yak1 in regulation of the stress-responsive transcription factors in response to nutrient starvation.

Chapter 6.

Regulation of yeast Yak1 kinase by PKA and autophosphorylation-dependent 14-3-3 binding

6.1. Introduction

The ability of proper adjustment to changes in the environmental conditions is a key process for cell survival. This adaptation is delicately coordinated by a variety of signal transduction networks that are sensitive to nutrient availability (Zaman *et al.*, 2008, Smets *et al.*, 2010). In eukaryotes, PKA (protein kinase A) and TOR (target of rapamycin) are two major nutrient-sensing kinases that promote cell growth through stimulation of protein synthesis, inhibition of stress responses and autophage, and regulation of other cellular processes (Thevelein & de Winde, 1999, De Virgilio & Loewith, 2006, Santangelo, 2006). The PKA and TOR pathways show a diverse ways of cross-talk, sharing several common targets through different regulatory mechanisms. Yak1 kinase in *Saccharomyces cerevisiae* is one such target which is negatively regulated by both PKA and TOR pathways (Schmelzle et al., 2004, Martin et al., 2004). Yak1 was initially identified as a growth antagonist based on the fact that deletion of YAK1 can suppress a growth defect of a strain lacking all three catalytic subunit of PKA, Tpk1, Tpk2, and Tpk3 (Garrett & Broach, 1989). Besides, Yak1 has been shown to be involved in thermotolerance (Hartley *et al.*, 1994)

and pseudohyphal growth (Zhang *et al.*, 2001, Goyard *et al.*, 2008, Iraqui *et al.*, 2005).

Yak1 is classified as a member of an evolutionarily conserved family of Ser/Thr protein kinases known as dual-specificity Tyr phosphorylation-regulated kinases (DYRKs), which are characterized by YXY motif in the activation loop in the catalytic domain (Kentrup et al., 1996, Yoshida, 2008). Autophosphorylation on the second Tyr residue in the YXY motif has been shown to be essential for full kinase activity of DYRKs including Yak1 (Kassis et al., 2000). After Tyr autophosphorylation, DYRKs act as strict Ser/Thr kinases for exogenous substrates. So far, several substrates of Yak1 kinase have been identified.

It has been shown that Yak1 can be directly phosphorylated by PKA *in vitro* and *in vivo*, suggesting that PKA might inhibit Yak1 through direct phosphorylation (Garrett et al., 1991, Zappacosta et al., 2002, Ptacek et al., 2005). Furthermore, subcellular localization of Yak1 mainly depends on nutrients, as Yak1 accumulates in the nucleus upon either glucose deprivation or inhibition of TOR pathway by rapamycin treatment (Moriya et al., 2001, Schmelzle et al., 2004, Martin et al., 2004). In addition, Yak1 have been shown to be associated with Bmh1 and Bmh1 only in the presence of glucose, eliciting the speculation that Bmh1/2 might play a role in nuclear

exclusion of Yak1 (Moriya et al., 2001). However, it has not yet been elucidated whether PKA and Bmh1/2 are involved in regulation of nucleocytoplasmic shuttling of Yak1.

In this chaper, differential role for PKA and Bmh1/2 in regulation of Yak1 was eludicated. It was demonstrated that PKA-dependent phosphorylation of Yak1, but not Bmh1/2 binding, inhibits its nuclear localization, whereas Bmh1 binds to Ser/Thr autophosphorylation site(s) of Yak1 and inhibits its catalytic activity. Considering the fact that Bmh1/2 binding to Yak1 coincides with PKA activity, such regulatory mechanisms might allow coordinated regulation of subcellular localization and kinase activity of Yak1 depending on glucose availability.

6.2 Mapping of PKA-dependent phosphorylation sites in Yak1

It has been shown that Yak1 is phosphorylated by bovine PKA (Garrett et al., 1991) and by Tpk1 and Tpk2, two out of three PKA catalytic subunits in *S. cerevisiae* (Ptacek et al., 2005), *in vitro*. In addition, mass spectrometry-based phosphopeptide mapping has revealed that Yak1 peptides containing five putative PKA phosphorylation sites

([R/K]$_2$XS/T) are phosphorylated *in vivo* (Zappacosta et al., 2002). However, it has not yet been demonstrated whether these putative sites are directly phosphorylated by PKA. Therefore, *in vitro* phosphorylation of Yak1 by Tpk1, one of the catalytic subunits of yeast PKA, using GST-tagged proteins purified from *E. coli* was investigated. The five putative PKA sites are located in the N-terminal domain of Yak1 (Fig. 6.1A). In agreement with their locations, the N-terminal domain of Yak1 (Yak1$_{1\text{-}368}$), but not the C-terminal domain (Yak1$_{369\text{-}807}$), was phosphorylated by GST-Tpk1 (Fig. 6.1B).

Among the five putative PKA sites, Ser127, Ser128, and Ser295 are located next to two putative nuclear localization signals, NLS1 (^{123}RRRK^{126}) and NLS2 (^{289}PKFRR^{293}) (Fig. 6.1A). Based on the previous observation that Yak1 translocates to the nucleus upon glucose starvation, it is possible that PKA might inhibit nuclear localization of Yak1. Therefore, it was mutated in the Ser127, Ser128, and Ser295 residues to Alanine, individually or in combination, and was examined Tpk1-dependent phosphorylation levels of the mutants. In order to eliminate the phosphorylation signal caused by autophosphorylation of Yak1, all the Yak1 mutants were generated in the context of Yak1^{K398R}, a kinase-inactive form of Yak1. As shown in Fig. 6.1C, Tpk1-dependent phosphorylation dec extent than Ser295. Additional mutation of all five putative PKA sites (Yak1^{5A}) did not show

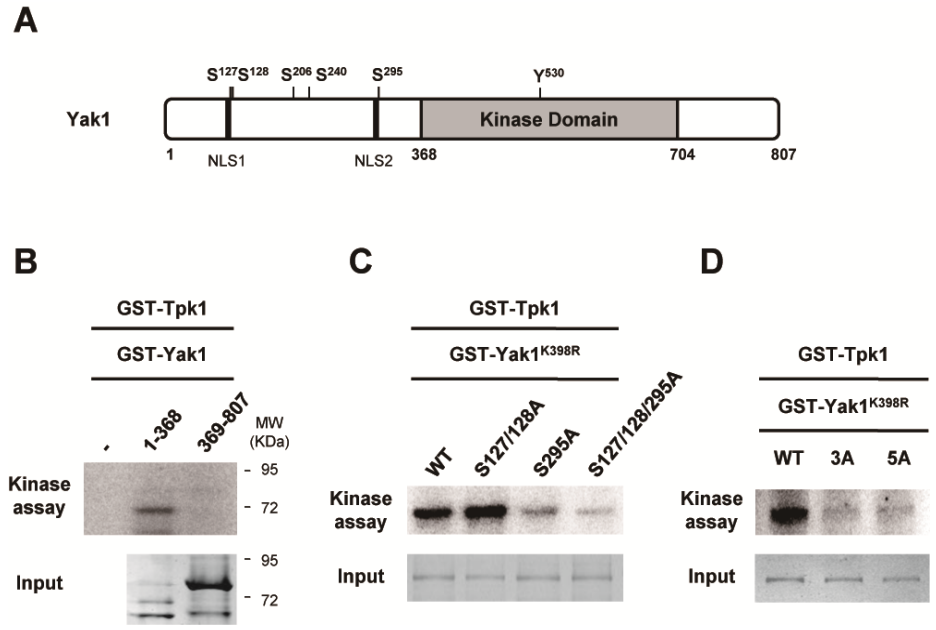

A
S127S128 S206 S240 S295 Y530
Yak1
Kinase Domain
1 NLS1 NLS2 368 704 807
B
GST-Tpk1
GST-Yak1
- 1-368 369-807 MW (KDa)
Kinase assay
Input
95
72
95
72
C
GST-Tpk1
GST-Yak1K398R
WT S127/128A S295A S127/128/295A
Kinase assay
Input
D
GST-Tpk1
GST-Yak1K398R
WT 3A 5A
Kinase assay
Input

Figure 6.1 Mapping of PKA-dependent phosphorylation sites in Yak1. (A) Schematic representation of Yak1 domain structure. The positions of Tyr autophosphorylation site in the catalytic loop (Y^{530}), five putative PKA phosphorylation sites (S^{127}, S^{128}, S^{206}, S^{240}, and S^{295}), and two putative nuclear localization signals (NLS1 and NLS2) are indicated. (B) Phosphorylation of the N-terminal domain of Yak1 by Tpk1 *in vitro*. GST-Tpk1 was purified from *E. coli* and *in vitro* kinase assay was performed using GST-$Yak1_{1-368}$ and GST-$Yak1_{369-807}$ proteins purified from *E. coli* as substrates. The GST-$Yak1_{1-368}$ and GST-$Yak1_{369-807}$ proteins used for the kinase assay are shown after silver staining (Input). (C and D). A catalytically inactive GST-$Yak1^{K398R}$ mutant (WT) or GST-$Yak1^{K398R}$ mutants of the indicated putative PKA phosphorylation sites were phosphorylated by GST-Tpk1 *in vitro*. 3A and 5A indicate mutations of three Ser residues (S^{127}, S^{128}, and S^{295}) and all five Ser residues shown in panel A, respectively. The same amount of mutant proteins were used as substrates as shown by silver staining (Input).

further reduction in phosphorylation level compared with Yak1^{3A}, suggesting that Ser206 and Ser240 are not well recognized by Tpk1 *in vitro* (Fig. 6.1D). Taken together, these results suggest that among the five putative PKA sites, Ser295 is the major site phosphorylated by Tpk1, with minor contributions of Ser127 and Ser128.reased significantly in the Yak1^{S295A} mutant, suggesting that S295 is the major site phosphorylated by Tpk1 in vitro. Although Yak1$^{S127/128A}$ showed a rather higher level of phosphorylation than wild type, mutation of all three sites (S127/128/295A) further reduced the phosphorylation level compared with the single S295A mutation. Therefore, Ser127 and Ser128 might also be phosphorylated by Tpk1, although to a lesser

6.3 PKA-dependent phosphorylation inhibits nuclear localization of Yak1

Next, it was tested whether the identified PKA-dependent phosphorylation sites are involved in regulation of subcellular localization of Yak1. In agreement with previous report, EGFP-Yak1 is accumulated in the nucleus upon glucose depletion (Fig. 6.2A) (Moriya et al., 2001). However, EGFP-Yak1^{3A}, harboring mutations at the three PKA sites, is mainly located in the nucleus even in the presence of

glucose, indicating that PKA-dependent phosphorylation is involved in retention of Yak1 in cytoplasm under high glucose conditions (Fig. 6.2A).

Rim15 kinase is responsible for G_o entry upon nutrient deprivation and shares some similarity with Yak1 in terms of regulation and function. Both kinases are negatively regulated by PKA and TOR, bind to Bmh1/2, and activate a same target, Msn2/4 (Swinnen *et al.*, 2006). Previously, it has been shown that kinase activity of Rim15 is required for its nuclear export by a yet unknown mechanism (Wanke et al., 2005). A catalytically inactive EGFP-Yak1^{K398R} mutant showed similar subcellular localization pattern to that of wild type depending of glucose availability (Fig. 6.2B). Therefore, kinase activity of Yak1 seems not to be essential for regulation of its subcellular localization. However, in the presence of glucose, EGFP-Yak1$^{K398R, 3A}$ (0.44 ± 0.09, nucleus/cytosoplasm) showed more distinct nuclear accumulation compared with EGFP-Yak1^{3A} (0.31 ± 0.06, nucleus/cytoplasm), implying that kinase activity of Yak1 might somehow contribute to its nuclear export as suggested for Rim15

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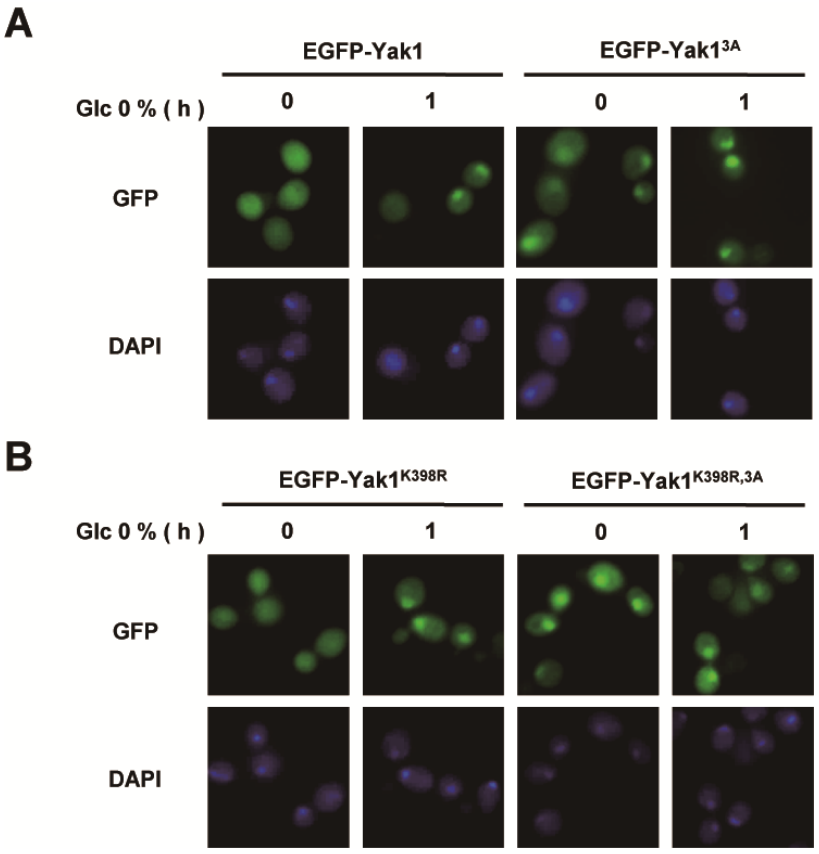

Figure 6.2 PKA-dependent phosphorylation inhibits nuclear localization of Yak1. DTY185/*yak1*⊿ cells expressing EGFP-Yak1, EGFP-Yak1^{3A} (A), EGFP-Yak1^{K398R}, or EGFP-Yak1^{K398R,3A} (B) were grown in a selective SC medium containing 4% glucose until early log phase and then shifted to a medium without glucose for 1 h. The subcellular localization of EGFP-Yak1 derivatives and DAPI-stained nucleus were observed by fluorescence microscopy.

6.4 Yak1 kinase activity, but not PKA-dependent phosphorylation, is necessary for Bmh1 binding

Yak1 has been reported to interact with Bmh1 and Bmh2, yeast 14-3-3 proteins, only in the presence of glucose (Moriya et al., 2001). 14-3-3 mainly binds to phosphorylated Ser/The residues, and one of the known functions of 14-3-3 is regulation of subcellular localization of its target proteins (van Heusden & Steensma, 2006). Therefore, it was asked whether PKA-dependent phosphorylation of Yak1 is necessary for Bmh1/2 binding. To answer this question, the interaction between Bmh1 and Yak1 by using yeast two-hybrid assay was detected (Fig. 6.3A) and His pull-down experiment by incubating His-Bmh1 purified from *E. coli* with either EGFP-Yak1 expressed in *S. cerevisiae* (Fig. 6.3B) or GST-Yak1 purified from *E. coli* (Fig. 6.3C). All three experiments showed that Yak1^{3A}, which cannot be phosphorylated by PKA, could bind to Bmh1 with an affinity similar to that of wild type Yak1. Furthermore, the fact that recombinant GST-Yak1 proteins purified from *E. coli*, where PKA activity does not exist, could bind to Bmh1 also supports the notion that Bmh1 does not bind to the Ser residues phosphorylated by PKA (Fig. 6.3C).

It has been shown that an autophosphorylation site of DYRK1A, one of the mammalian homologues of Yak1, mediates interaction of DYRK1A with 14-3-3 (Alvarez *et al.*, 2007). Therefore, it was investigated whether kinase activity of Yak1 is also required for Bmh1 binding. As shown in Fig. 6.3, Yak1^{K398R} failed to interact with Bmh1 both *in vivo* and *in vitro*, suggesting that the autophosphorylation site of Yak1 might provide a binding site for Bmh1. Note that GST-Yak1^{K398R} purified from *E. coli* has faster mobility on SDS-PAGE than wild type GST-Yak1, which implies that autophosphorylation of GST-Yak1, but not GST-Yak1^{K398R}, can occur when expressed in *E. coli*.

In addition, the fact that EGFP-Yak1^{K398R}, which cannot bind to Bmh1, showed normal cytoplasmic localization in the presence of glucose suggests that Bmh1 binding is not critical for nuclear exclusion of Yak1 (Fig. 6.2A). However, since EGFP-Yak1$^{K398R,\ 3A}$ showed a stronger nuclear localization signal than EGFP-Yak1^{3A} under high glucose conditions, it cannot be ruled out the possibility that Bmh1 binding can contribute to nuclear export of Yak1.

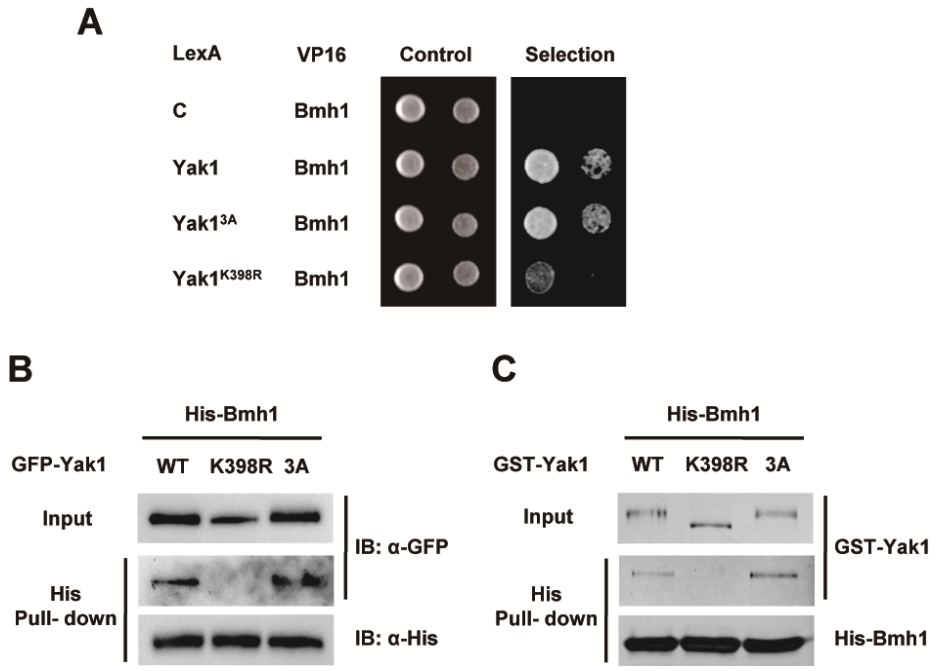
A
LexA
VP16
Control
Selection
C
Bmh1
Yak1
Bmh1
Yak1^3A
Bmh1
Yak1^K398R
Bmh1
B
His-Bmh1
GFP-Yak1
WT
K398R
3A
Input
His
Pull- down
IB: α-GFP
IB: α-His
C
His-Bmh1
GST-Yak1
WT
K398R
3A
Input
His
Pull- down
GST-Yak1
His-Bmh1

Figure 6.3 Yak1 kinase activity, but not PKA-dependent phosphorylation, is necessary for Bmh1 binding. (A) Yeast two-hybrid interactions of Yak1 derivatives and Bmh1. *S. cerevisiae* strain L40-Ura cells expressing LexA-fused Yak1, Yak1^{K398R}, or Yak1^{3A} and VP16-fused Bmh1 were spotted on a control plate medium containing His and a selection medium containing 50 mM 3-AT but lacking His to detect protein-protein interaction. (B) DTY185/*yak1⊿* cells were transformed with plasmids expressing EGFP-Yak1, EGFP-Yak1^{K398R}, or EGFP-Yak1^{3A}, and the cell lysates were incubated with His-Bmh1 proteins purified from *E. coli.* After His pull-down experiment using Ni^{2+}-NTA agarose, the samples were analyzed by Immunoblotting. (C) Interaction between Yak1 and Bmh1 *in vitro*. His-Bmh1 proteins and the indicated GST-Yak1 derivatives purified from *E. coli* were incubated with Ni^{2+}-NTA agarose, and the precipitates were analyzed by silver staining.

6.5 Intramolecular Ser/Thr autophosphorylation of Yak1 is necessary for its kinase activity

Since autophosphorylation of Yak1 seemed to be a prerequisite for Bmh1 binding, the autophosphorylation reaction of Yak1 was further investigated. As shown in Fig. 6.4A, the N-terminal domain of Yak1 ($Yak1_{1-368}$), but not the C-terminal domain ($Yak1_{369-807}$), was phosphorylated by GST-Yak1 *in vitro*. The $Yak1_{1-368}^{5A}$ mutant, lacking all five PKA sites, was phosphorylated to a similar level to that of wild type $Yak1_{1-368}$, suggesting that Yak1 autophosphorylates its N-terminal domain on sites different from the PKA phosphorylation sites. In order to elucidate whether autophosphorylation of Yak1 occurs through intermolecular or intramolecular reaction, His-Yak1 and GST-$Yak1^{K398R}$, which exhibit different mobility on SDS-PAGE were used. Whereas GST-$Yak1^{K398R}$ was phosphorylated by Tpk1 *in vitro*, when GST-$Yak1^{K398R}$ was incubated with His-Yak1, phosphorylation of GST-$Yak1^{K398R}$ was not detected, exhibiting only autophosphorylated His-Yak1 band (Fig. 6.4B). The fact that His-Yak1 cannot phosphorylate GST-$Yak1^{K398R}$ indicates that autophosphorylation of Yak1 is an intramolecular event, although Yak1 can phosphorylate the truncated

N-terminal domain of Yak1 as an exogenous substrate through intermolecular phosphorylation.

Previous mass spectrometry-based phosphopeptide mapping revealed at least eleven phosphorylation sites in Yak1 in vivo, including five putative PKA sites and Tyr autophosphorylation site (Tyr530) in the activation loop (Fig. 6.1A). Among the five remaining sites, four residues, Ser234, Ser247, Thr288, and Ser302, are followed by Pro (Fig. 6.4C). Previously, these sites were predicted to have cyclin-dependent kinase (CDK) consensus motifs, S/TP. Recently, RXXS/TP was suggested as a Yak1 consensus recognition motif although Pro at +1 position showed rather weak selectivity (Mok *et al.*, 2010). Therefore, these Ser or Thr residues as potential autophosphorylation sites of Yak1 were considered. In addition, although it was not detected by previous phosphopeptide analysis, it was identified RVLT^{335}KP motif which is similar to the 14-3-3 mode I binding motif (RSXpS/pTXP) as well as satisfying the Yak1 phosphorylation consensus sequence (Fig. 6.4C). In order to verify these potential autophosphorylation sites, each Ser or Thr residue to Ala was mutated, and its effect on *in vitro* autophosphorylation and kinase activity toward Hsf1$_{1\text{-}155}$, one of the known substrate of Yak1 were examined. Yak1^{S247A} was excluded from the experiment because of its instability during purification from *E. coli*. As shown in Fig. 6.4D, all four Yak1 mutants tested, S234A, S288A,

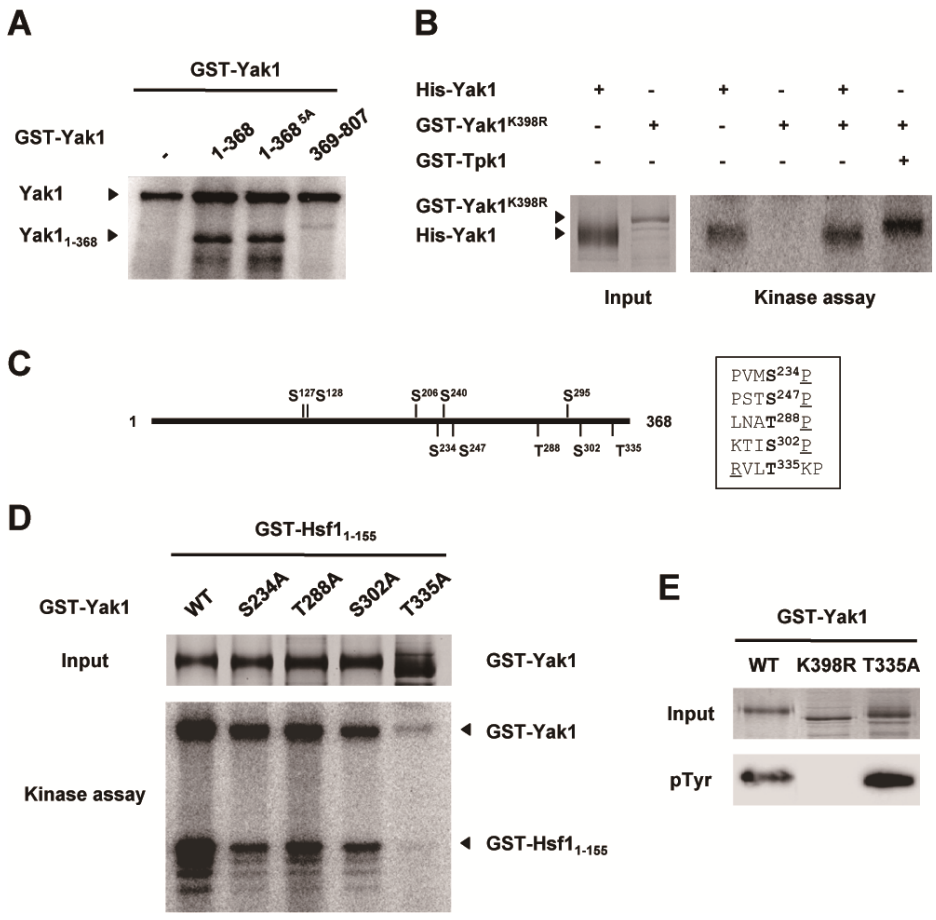
A
GST-Yak1
GST-Yak1
-
1-368
1-368 5A
369-807
Yak1
Yak1 1-368
B
His-Yak1
GST-Yak1 K398R
GST-Tpk1
GST-Yak1 K398R
His-Yak1
Input
Kinase assay
C
1
368
S127 S128
S206 S240
S295
S234 S247
T288
S302
T335
PVMS234P
PSTS247P
LNAT288P
KTIS302P
RVLT335KP
D
GST-Hsf1 1-155
GST-Yak1
WT
S234A
T288A
S302A
T335A
Input
Kinase assay
GST-Yak1
GST-Yak1
GST-Hsf1 1-155
E
GST-Yak1
WT
K398R
T335A
Input
pTyr

Figure 6.4 Intramolecular Ser/Thr autophosphorylation of Yak1 is required for its kinase activity. (A) Autophosphorylation of the N-terminal domain of Yak1 *in vitro*. Kinase assays were performed with GST-Yak1 using GST-Yak1$_{1\text{-}368}$ GST-Yak1$_{1\text{-}368}{}^{5A}$, or GST-Yak1$_{369\text{-}807}$ as substrates. (B) Yak1 autophosphorylates through intramolecular reaction. GST-Yak1^{K398R} was phosphorylated by His-Yak1 or GST-Tpk1 *in vitro*. (C) Putative autophosphorylation sites in Yak1. PKA phosphorylation sites and the putative Yak1 autophosphorylation sites are shown above and below the bar, respectively. The amino acid sequences around the putative autophosphorylation sites are shown in a box. The residues matching with the Yak1 recognition motif (RXXS/TP) are underlined. (D) Yak1 autophosphorylation activates its kinase activity. GST-Yak1 proteins, each containing a mutation at the indicated autophosphorylation site, were purified from *E. coli*, and the levels of autophosphorylation and phosphorylation of GST-Hsf1$_{1\text{-}155}$ were detected by kinase assay. (E) Mutation of the autophosphorylation site, Thr335, does not affect Tyr autophosphorylation. Wild type and mutant GST-Yak1 proteins were purified from *E. coli* and their levels of Tyr autophosphorylation were detected by immunoblotting with anti-phosphotyrosine antibody. The proteins used for the experiment are shown after staining with coomassie brilliant blue (input).

S302A, and T335A, showed reduced levels of both autophosphorylation and phosphorylation of their substrate. Especially, Yak1^{T335A} mutant showed the most dramatic reduction in autophosphorylation and kinase activity. The faster mobility of Yak1^{T335A} on SDS-PAGE compared with other mutants also supports the notion that Thr335 is the major autophosphorylation site. However, Tyr autophosphorylation level, detected by anti-phosphotyrosine antibody, was not changed by T335A mutation, indicating that Tyr autophosphorylation in the activation loop is an earlier event than Ser/Thr autophosphorylation, but Tyr autophosphorylation itself is not enough for full kinase activity of Yak1 (Fig. 6.4E). Taken together, these results demonstrate that in addition to the previously known Tyr autophosphorylation, intramolecular Ser/Thr autophosphorylation of the Yak1 N-terminal noncatalytic domain, especially on Thr335, is critical for the kinase activity of Yak1.

6.6 Bmh1 binds to the phosphorylated N-terminal domain of Yak1

The interaction between Yak1 and Bmh1 by mapping the Bmh1 binding sites in Yak1 was further investigated. Yeast two-hybrid

analyses using serial Yak1 deletion mutants revealed that the N-terminal domain of Yak1 ($Yak1_{1-368}$), where Ser/Thr autophosphorylation sites are located, is engaged in Bmh1 binding (Fig. 6.5A). Deletion of the N-terminal 200 amino acids of Yak1 diminished its interaction with Bmh1 and further deletion up to 300 amino acids almost abolished the interaction (Fig. 6.5A).

The interaction between Bmh1 and Yak1 by in vitro pull-down assays using His-Bmh1 and a series of truncated mutants of GST-Yak1 purified from *E. coli* was also examined. As shown in Fig. 6.5B, *in vitro* pull-down assays showed similar results to those obtained from yeast two-hybrid assays. GST-$Yak1_{201-807}$ showed slightly reduced interaction with His-Bmh1 compared with full length GST-Yak1 ($Yak1_{1-807}$), whereas GST-$Yak_{301-807}$ failed to bind to His-Bmh1. Therefore, both *in vivo* and *in vitro* interaction data commonly suggest the essential role of the N-terminal region between 200 and 300 amino acid residues for Bmh1 binding.

However, in contrast to the strong binding of $Yak1_{1-368}$ to Bmh1 in yeast two-hybrid assay, interaction between these proteins was not detected by the in vitro His pull-down experiment (Fig. 6.5A and 5B). These apparently contradictory results could be reconciled by the fact that Bmh1 recognizes phosphorylated residues in Yak1. Although $Yak1_{1-368}$ can be phosphorylated by endogenous Yak1 in yeast cells, it

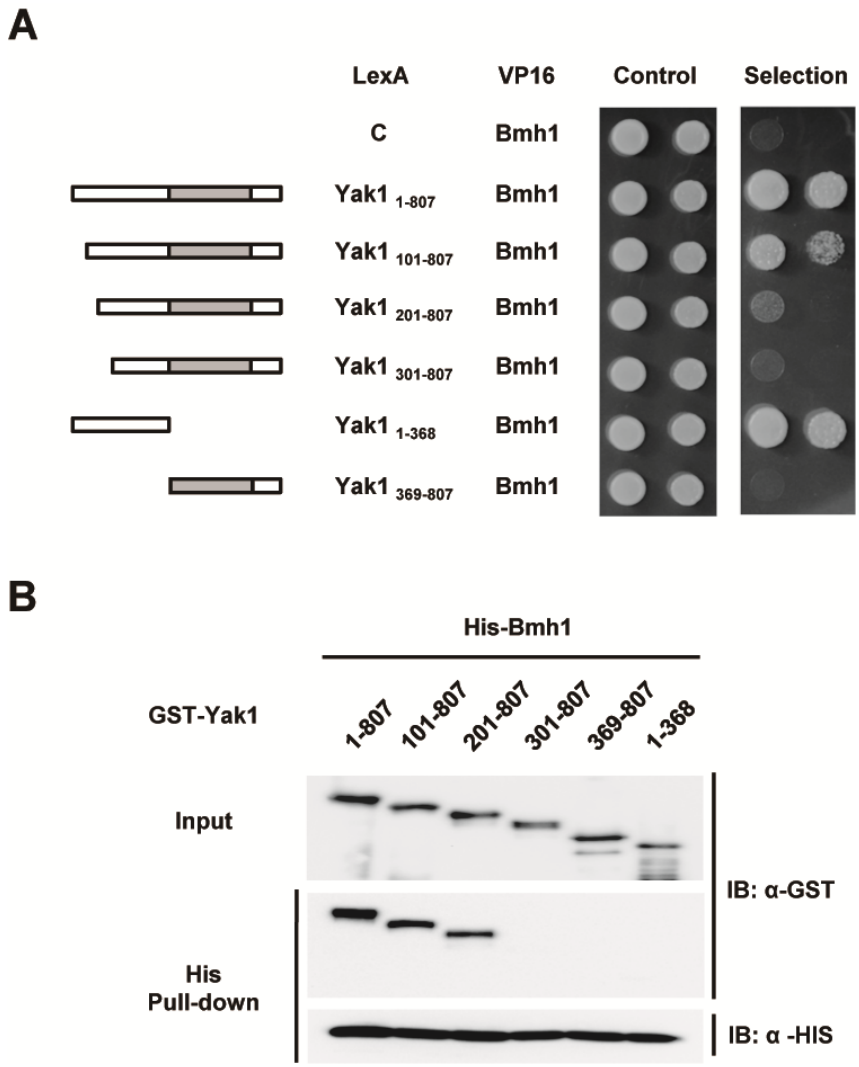
A
LexA
VP16
Control
Selection
C
Bmh1
Yak1 1-807
Bmh1
Yak1 101-807
Bmh1
Yak1 201-807
Bmh1
Yak1 301-807
Bmh1
Yak1 1-368
Bmh1
Yak1 369-807
Bmh1
B
His-Bmh1
GST-Yak1
1-807
101-807
201-807
301-807
369-807
1-368
Input
His
Pull-down
IB: α-GST
IB: α -HIS

Figure 6.5 Binding of Bmh1 to the phosphorylated N-terminal domain of Yak1. (A) Mapping of interaction domains between Yak1 and Bmh1 in vivo. Interaction between the indicated Yak1 derivatives and Bmh1 was detected by yeast two-hybrid assays. (B) Mapping of interaction domains between Yak1 and Bmh1 in vitro. His-Bmh1 was incubated with GST-Yak1 or truncated GST-Yak1 variants purified from E. coli, and then the samples were subjected to His pull-down assays followed by immunoblotting.

cannot be phosphorylated during purification in *E. coli*. Since GST-$Yak_{101-807}$, GST-$Yak_{201-807}$, and GST-$Yak_{301-807}$ proteins contain kinase domain, these proteins can be autophosphorylated even if purified from *E. coli*, which was confirmed by accelerated migration on SDS-PAGE upon alkaline phosphatase treatment, and by detection of Tyr autophosphorylation using anti-phosphotyrosine antibody (data not shown).

6.7 Autophosphorylation of Yak1 on T335 is necessary for Bmh1 binding

Based on the fact that autophosphorylation of Yak1 is critical for Bmh1 binding, the role of each autophosphorylation site for Bmh1 binding was next examined. Among the Yak1 autophosphorylation site mutants tested, only $Yak1^{T335A}$ failed to interact with Bmh1 in yeast two-hybrid assays (Fig. 6.6A). These results are consistent with that fact that Thr335 is the major autophosphorylation site in Yak1 (Fig. 6.4D).

The critical role of Thr335 phosphorylation for Bmh1 binding by in vitro pull-down assay was also confirmed. A GST-$Yak1^{T335A}$ mutant showed significant reduction in binding affinity to His-Bmh1 compared with wild type Yak1 (Fig. 6.6B). However, although $Yak1^{K398R}$ almost

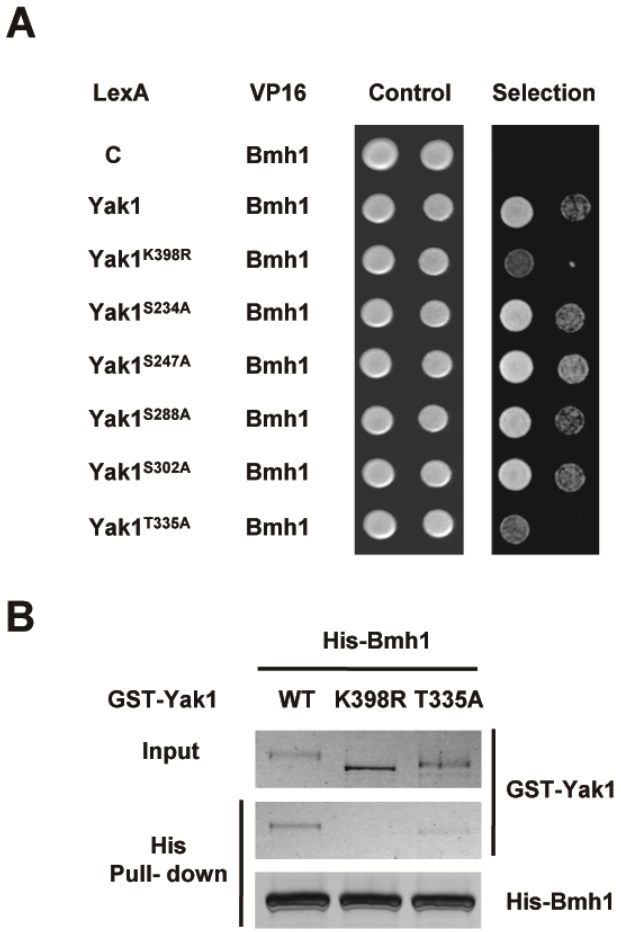

Figure 6.6 Autophosphorylation of Yak1 on Thr335 is necessary for interaction between Yak1 and Bmh1. (A) Yeast two-hybrid assays were performed with the indicated Yak1 variants and Bmh1. (B) His pull-down experiments were performed using His-Bmh1 and the indicated GST-Yak1 mutants purified from *E. coli*. Proteins were detected by silver staining

lost its binding affinity to Bmh1, Yak1^{T335A} could still bind to Bmh1 with low affinity. These results suggest that although phosphorylated Thr335 plays a major role for Bmh1 binding, the remaining autophosphorylation site(s) might also contribute to Bmh1 binding. The fact that Yak1$_{301-807}$, which contains Thr335 residue, could not bind to Bmh1 both *in vivo* and *in vitro*, also support the requirement of additional binding site for Bmh1 (Fig. 6.5). Considering the fact that Bmh1 works as a dimer, phosphorylated Thr335 might provide a primary binding site for one Bmh1 subunit, but this binding only might not be strong enough to support stable binding of a Bmh1 dimer. Binding of the other Bmh1 monomer to a second binding site in the N-terminal 200 to 300 amino acid residues might be necessary to stabilize the interaction.

6.8 Bmh1 binding inhibits kinase activity of Yak1

In this chapter, it was shown that Bmh1 binds to at least one Yak1 autophosphorylation site that is essential for its kinase activity. Therefore, it was examined whether kinase activity of Yak1 can be modulated by Bmh1 binding. As a control, it was generated a His-Bmh1^{K51E} mutant having a mutation at Lys51 that is a conserved

critical residue in the target binding motif of 14-3-3 (Kim *et al.*, 2004, Bridges & Moorhead, 2005). As shown in Fig. 6.7A, His-Bmh1, but not His-Bmh1^{K51E} mutant, was pulled down by GST-Yak1 *in vitro*.

Next, *in vitro* kinase activity of GST-Yak1 in the presence of various amount of recombinant His-Bmh1 or His-Bmh1^{K51E} proteins was investigated. As shown in Fig. 6.7B, autophosphorylation of Yak1 was inhibited by His-Bmh1 in a dose-dependent manner. Addition of 30 μM His-Bmh1 caused approximately 24% inhibition of Yak1 autophosphorylation. Phosphorylation of its substrate, GST-Hsf1$_{1\text{-}155}$, was also decreased by Bmh1 binding, exhibiting approximately 32% inhibition in the presence of 30 μM His-Bmh1 (Fig. 6.7B). However, Bmh1^{K51E}, which cannot bind to Yak1, did not affect Yak1 autophosphorylation nor substrate phosphorylation (Fig. 6.7B). In summary, these findings suggest that Bmh1 inhibits kinase activity of Yak1 by binding to the Yak1 autophosphorylation site that is essential for its kinase activity.

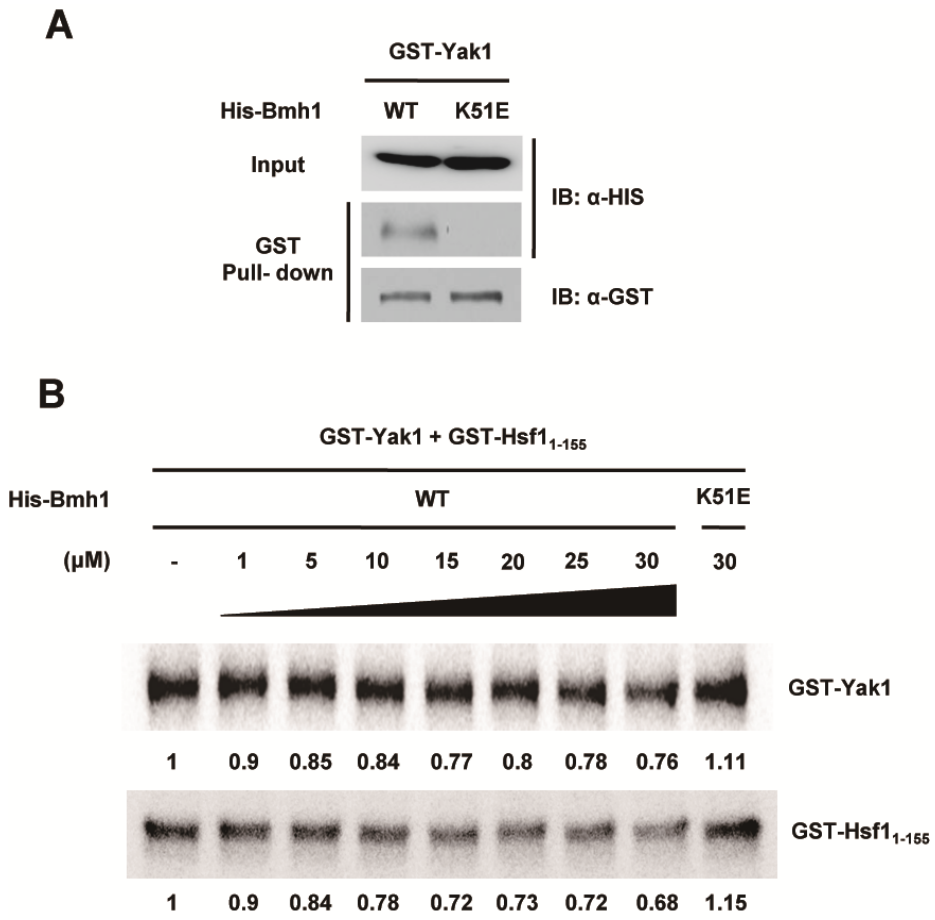
A
GST-Yak1
His-Bmh1
WT
K51E
Input
IB: α-HIS
GST
Pull- down
IB: α-GST
B
GST-Yak1 + GST-Hsf1 1-155
His-Bmh1
WT
K51E
(μM)
-
1
5
10
15
20
25
30
30
GST-Yak1
1
0.9
0.85
0.84
0.77
0.8
0.78
0.76
1.11
GST-Hsf1 1-155
1
0.9
0.84
0.78
0.72
0.73
0.72
0.68
1.15

Figure 6.7 Bmh1 binding inhibits kinase activity of Yak1. (A) His-Bmh1, but not His-Bmh1^{K51E}, binds to GST-Yak1 *in vitro*. GST-Yak1 was incubated with His-Bmh1 or His-Bmh1^{K51E} mutant, and the precipitates with glutathione-agarose were analyzed by Immunoblotting. (B) Levels of autophosphorylation and phosphorylation of GST-Hsf1$_{1-155}$ were measured in the presence of the indicated amounts of His-Bmh1 or His-Bmh1^{K51E}. Phosphorylation levels were quantified by phosphorimager and the relative values normalized to a control without Bmh1 were indicated below each band.

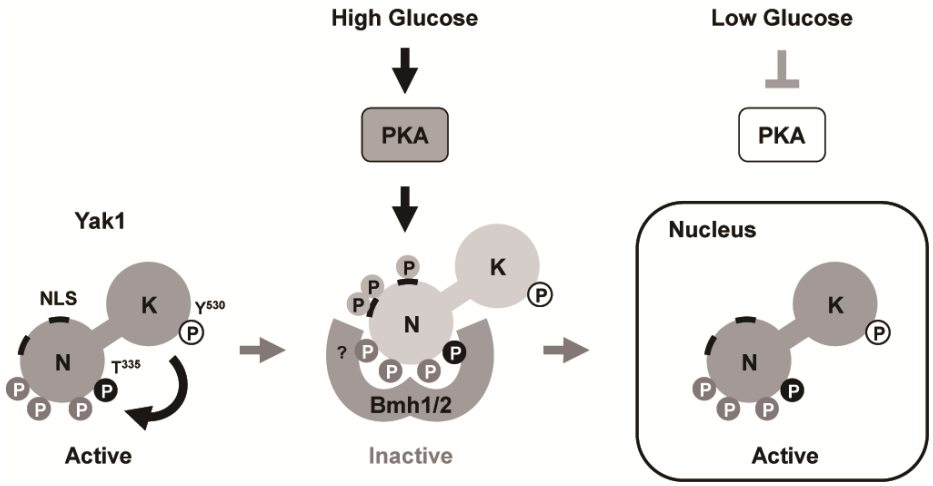

Figure 6.8 Model for the regulation of Yak1 by PKA and Bmh1/2. Yak1 kinase autophosphorylates on Tyr530 in the catalytic domain (K) and Ser or Thr residues in the N-terminal domain (N), which is essential for its kinase activity. Under high glucose conditions, the autophosphorylated Thr335 residue serves as a primary site for Bmh1/2 binding in collaboration with a yet unidentified second binding site in the N-terminus, inhibiting the kinase activity of Yak1. In addition, PKA-dependent phosphorylation masks the putative nuclear localization signals (NLS) of Yak1, thus retaining an inactive form of Yak1 in cytoplasm. Upon glucose starvation, Inactivation of PKA leads to nuclear import of Yak1, and release of Bmh1/2 from Yak1 by a yet unknown mechanism restores kinase activity of Yak1.

6.9 Conclusions

In this chapter, it has been shown that Bmh1, a yeast homologue of 14-3-3 proteins, is involved in the inactivation of Yak1 kinase via direct binding. Furthermore, this is the first report demonstrating the requirement of Ser/Thr autophosphorylation, not the Tyr autophosphorylation, for full kinase activity of Yak1 in *Sacchromyces cerevisiae*. These results suggest a novel Ser/Thr autophosphorylation-dependent activation mechanism of Yak1 and its regulation of nucleocytoplasmic shuttling and kinase activity by PKA and Bmh1/2, respectively. This study includes biologically important and novel findings, which would contribute to the understanding of differential regulatory mechanism for PKA and Bmh1/2 in regulation of Yak1 kinase.

Chapter 7.

Identification of substrate specificity of Yak1 kinase using high-throughput phosphorylation profiling method (HTTP)

7.1. Introduction

Protein phosphorylation by protein kinase is regarded as the most widespread post-translational modification in signal transduction pathways (Manning *et al.*, 2002). Identification of the substrate specificity of protein kinases is important not only to understand the complicated signaling pathways but also to develop durgs against protein kinases involved in human diseases. Therefore, screening of the substrate specificity of Ser/Thr kinases is important to understand their biological functions. Although serveral methods including combinatorial peptide microarrays and positional-scanning peptide library has been developed (Lesaicherre *et al.*, 2002, Schutkowski *et al.*, 2005, Gast *et al.*, 1999, Hutti *et al.*, 2004, Turk *et al.*, 2006), these approches have disadvantages in identifying the complete substrate pepetide sequences of kinases. To overcome this difficulty, 'high-throughput phosphorylating profiling (HTPP)' method has been developed for high-throughput identification of substrate specificity of Ser/Thr kinases using a fully-randomized one-bead one-compund (OBOC) combinatorial ladder type peptide library and MALDI-TOF MS (Lam *et al.*, 1997, Lam *et al.*, 1991). The advantage of HTPP method is that only one reaction using the target kinase is enough to obtain the

potential substrates. Moreover, the complete substrate sequence is obtained from the ladder peptide library, which is sufficient to cover all the variations of amino acids at each randomized position using developed HTPP method.

In this chapter, using high-throughput phosphorylation profiling (HTPP) method, the substrate specificity of Yak1 was determined. Furthermore, Ifh1, a co-activator for the transcription of ribosomal protein genes, was newly identified as a Yak1 target.

7.2. Substrate specificity of Yak1 kinase

For last decade, several substrates of Yak1 kinase has been found including Pop2 regulating mRNA deadenylation (Moriya et al., 2001), Msn2 and Hsf1 transcription factors for stress response genes (Lee et al., 2008), and Crf1 co-repressor for the expression of ribosomal protein genes (Martin et al., 2004). However, its substrate specificity has not yet been completely characterized. Therefore, the substrate spepcificity of Yak1 kinase was elucidated by HTPP method (Fig 7.1). Subsequent to the use of HTPP method, 97 peptide sequences out of 100 red-colored beads (97%) could be identified from MALDI-TOF spectra. The eluted ladder peptides from one bead could be

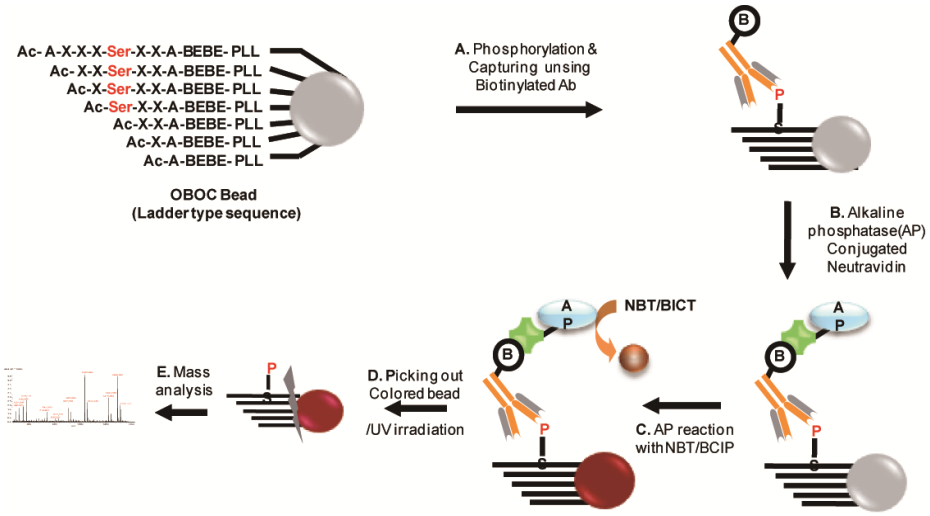

Figure 7.1 HTPP procedure for determination of Yak1 kinase substrate specificity. (A) The phosphorylation and biotinylation step. Phosphorylation of peptide library beads is performed by Yak1 kinase. After the kinase reaction, phosphoserine peptides were incubated with biotin-conugated antiphosphoserine Ab. (B) Capturing step of the biotinylated peptide beads containing phosphoserine. AP-conjugated Neutravidin is added and reaction mixture is further incubated. (C) Detection step. After the reaction of AP, the color of phsphorylated beads changes to dark-red. (D) Picking up step. Eluted from one bead were analyzed by MALDI-TOF. The peptide was sequenced by mass difference of each peptide peak.

sequenced by calculating mass differences corresponding to each amino acid. Among the obtained peptide sequences, peptide containing Arg at P (-3) is the only dominant residue identified by this screening method. Therefore, the substrate motif of Yak1 kinase can be determined to be RXXS.

7.3. Identification of Ifh1 as a new substrate of Yak1

Among the obtained peptide sequences, peptides having Arg residue at P (-3) were compared with the *S. cerevisiae* database using the BLAST search and the peptide that matched well was selected. The three peptides of RRFSAF (Msn2), RKDSGI (Msn4), and RHKSGK (Ifh1) matched well with sequences in potential substrate proteins of Yak1. Especially, the RHKSGK peptide sequence perfectly matched with amino acid residues 519-524 of Ifh1, which is a co-activator for the transcription of ribosomal protein genes. Ifh1 competes with Crf1 as co-repressor in binding to Fhl1. It has been shown previously that Crf1 is directly phosphorylated by Yak1.

It was further attempted to confirm the newly identified Yak1 substrate protein, Ifh1, by *in vitro* kinase assay. Msn2 and Msn4 the known substrates of Yak1 were used as positive controls for *in vitro*

kinase assay. Msn2 (401-704), Msn4 (1-630), Ifh1 (401-800) containing the potential phosphorylation sites (RRFSAF, RKDSGI, and RHKSGK), but not Ifh1 (1-400), were phosphorylated by Yak1 (Fig 7.2A).

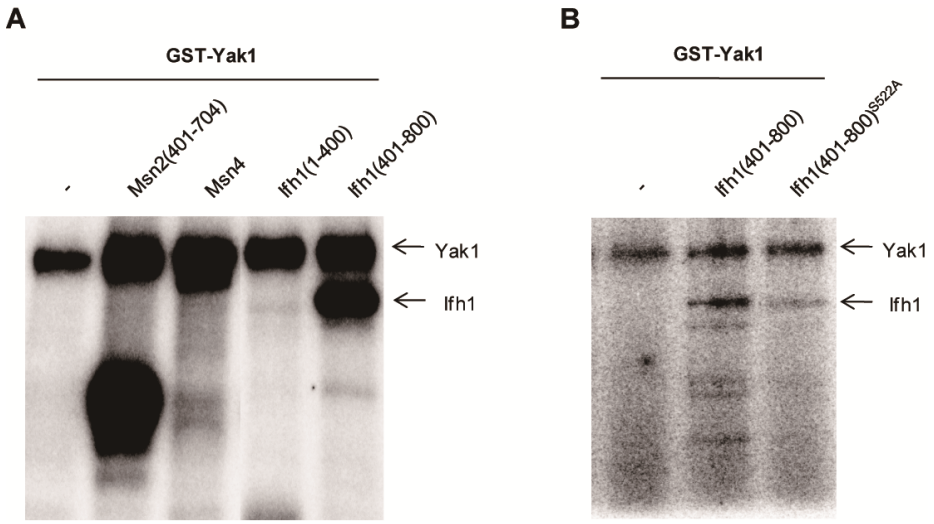

Figure 7.2 Substrates of Yak1-dependent phosphorylation. (A) GST-Yak1 was purified in *E.coli* and *in vitro* kinase assays were performed using GST-Msn2 (401-704), GST-Msn4, GST-Ifh1 (1-400), and GST-Ifh1 (401-800). Autophsphorylation of Yak1 and phosphorylation of proteins were detected by SDS-PAGE followed by autoradiography. (B) GST-Ifh1 (401-800) wiled type and S522A mutant were phosphorylated by GST-Yak1 *in vitro*.

To confirm the phosphorylation site, Ser522 of Ifh1 (401-800) was replaced with Ala. In agreement with the prediction, the S522A mutant showed reduced phosphorylation by Yak1 (Fig 7. 2B).

7.4. Conclusions

In this chapter 7, substrate specificity of Yak1 kinase was demonstrated by high throughput phosphorylation profiling (HTPP) method. RXXS was determined as a consensus phosphorylation motif of Yak1 kinase. In addition, Ifh1, was identified as a novel substrate of Yak1 kinase. Therefore, the HTPP method could serve as a useful tool to understand the substrate specificity and fuction of Ser/Thr kinases.

Chapter 8.

Overall Discussion and Further Suggestions

Hsf1 is activated by Yak1-dependent phosphorylation under the conditions of low PKA activity

Heat shock transcription factor (HSF), which activates gene expression in response to stress, plays an important role not only in stress resistance but also in cell growth and development (Morimoto, 1998, Pirkkala *et al.*, 2001). Although heat shock is the best studied activation signal for HSF, it becomes evident that HSF can be activated by a wide range of stress conditions including oxidative stress, heavy metals, and nutrient starvation. While mammalian HSF1, the stress-responsive mammalian HSF isoform, is regulated at multiple levels including homotrimerization, nuclear accumulation, DNA binding, and transactivation (Morano & Thiele, 1999, Wu, 1995, Pirkkala et al., 2001, Morimoto, 1998), yeast Hsf1 is always in the nucleus as a trimer and is engaged in both constitutive and stress-inducible binding to HSEs (Sorger *et al.*, 1987, Hoj & Jakobsen, 1994, Erkine *et al.*, 1999, Hahn et al., 2004). Activation of Hsf1 is thought to be induced by conformational changes triggered either by direct sensing of elevated temperature, oxidants, or low pH (Ahn & Thiele, 2003, Zhong *et al.*, 1998, Zhong *et al.*, 1999), or by covalent modifications such as phosphorylation. Phosphorylation is a common regulatory mechanism

shared by all HSFs from yeast to humans. Activity of mammalian HSF1 has been shown to be repressed by several kinases such as GSK-3, ERK, PKC, JNK, whereas activated by CaMKII-dependent phosphorylation (Holmberg *et al.*, 2002, Holmberg et al., 2001). Yeast Hsf1 is also regulated by multiple phosphorylations in both negative and positive ways. Phosphorylation of serines adjacent to a heptapeptide region (CE2) of the yeast *Kluyveromyces lactis* Hsf1 has been shown to restrain Hsf1 in an inactive state (Hoj & Jakobsen, 1994). On the other hand, induction of differential hyperphosphorylation under different stress conditions has been suggested as a mechanism for stress-specific activation of Hsf1, which has been supported by the identification of Snf1 kinase that phosphorylates and activates Hsf1 in response to glucose starvation without affecting heat shock-induced activation of Hsf1 (Hahn & Thiele, 2004).

As an effort to understand the role of differential phosphorylation in regulation of Hsf1 activity, 136 putative protein kinases in *S. cerevisiae* genome were screened (Hunter & Plowman, 1997) and identified five kinases (Snf1, Cmk1, Cmk2, Kns1, and Yak1) which can phosphorylate Hsf1 *in vitro*. it has been demonstrated that Yak1, whose expression and activity is repressed by PKA, can activate Hsf1 at the level of DNA binding by direct phosphorylation under the

conditions of low PKA activity, reinforcing the proposed role for phosphorylation in stress-specific activation of Hsf1. Recently, it has been shown that PKA inhibits Hsf1 activity under normal conditions (Ferguson et al., 2005) or upon treatment of menadione, a superoxide-generation reagent (Yamamoto *et al.*, 2007). Our results suggest that Yak1 might be the mediator of PKA-dependent regulation of Hsf1, but the residual activation of Hsf1 in yak1Δ indicates that Yak1 may not be the only downstream kinase of PKA involved in Hsf1 activation.

Although both Snf1 and Yak1 activate Hsf1 in response to glucose limitation, their roles seem to be differentiated to adapt to specific physiological conditions. Under the conditions of acute glucose depletion, mainly Yak1, but not Snf1 is responsible for Hsf1 activation. These results might reflect different signal transduction pathways for activation of Snf1 and Yak1 and interactions with other regulatory networks, which are not completely understood yet. In addition, although both Snf1- and Yak1-dependent phosphorylation have been shown to enhance DNA binding activity of Hsf1 (Hahn & Thiele, 2004), these kinases seem to phosphorylate different sites in Hsf1. The differential phosphorylation might induce slightly different conformational changes in Hsf1, allowing activation of different subset of genes depending on the architecture of HSEs. The target genes

largely affected by Yak1- and Snf1-dependent phosphorylation of Hsf1 in terms of DNA binding activity seem to be the genes with weak HSEs which cannot provide constitutive binding of Hsf1 under normal conditions. It has been shown that Snf1-dependent glucose starvation-inducible binding of Hsf1 in vivo is noticeable for the promoters with low basal Hsf1 binding affinities such as *SSA3* and *HSP30* (Hahn & Thiele, 2004). Our *in vitro* DNA binding assay also revealed that although Yak1-dependent phosphorylation increased DNA binding activity of Hsf1 even to strong HSE with five perfect inverted repeat of nGAAn unit, the phosphorylation-dependent fold decrease in K_d was higher for HSEs with weak Hsf1 binding affinity such as gap type HSE. However, significant HSE type-specificity in Yak1-dependent expression *in vivo* was not observed. Expression levels HSE reporters were reduced in *yak1Δ* to a similar extent independent of HSE types. The differential effect of Yak1 on Hsf1 DNA binding activity to different HSE types observed *in vitro* might not be large enough to elicit differential transcription *in vivo*. In addition, although our in vitro DNA binding assay has clearly demonstrated the role for phosphorylation in increasing DNA binding activity of Hsf1, it cannot be ruled out the possibility that phosphorylation can play an additional role in regulating Hsf1 transactivation activity after DNA binding. Identification of the

phosphorylation sites of Hsf1 and any subsequent changes in promoter specificity would be necessary to understand the precise roles for Snf1- and Yak1-dependent phosphorylation of Hsf1.

Msn2 is activated by Yak1-dependent phosphorylation

It has been demonstrated that Yak1 activates not only Hsf1 but also Msn2/4 possibly by direct phosphorylation. So far, two essential stress-responsive transcription factors, Hsf1 and Msn2, are considered as independent regulators in terms of signal transduction pathways for their activation. Our study demonstrates for the first time that PKA can coordinately inhibit these transcription factors via regulating downstream kinase Yak1. It has been well documented that PKA inhibits Msn2 by phosphorylating four PKA sites within NLS of Msn2 (Gorner et al., 2002, De Wever et al., 2005). In this study, it has been shown that nuclear translocation is not sufficient for full activation of Msn2/4 and there exist another indirect mechanism for PKA-dependent regulation of Msn2/4 via Yak1. Although it is not clear yet whether PKA-dependent phosphorylation of Yak1 is involved in inhibiting nuclear translocation of Yak1, both Msn2 and Yak1 are phosphorylated by PKA and translocated to the nucleus upon glucose

depletion (Gorner et al., 1998, Gorner et al., 2002, Smith et al., 1998, Schmelzle et al., 2004, Moriya et al., 2001). Yak1 then may phosphorylate and activate Msn2 through yet unidentified mechanism (Fig. 4.7). Yak1-dependent phosphorylation of Msn2 did not affect DNA binding activity of C-terminal domain of Msn2, implying that Yak1 might regulate Msn2 activity after the event of DNA binding. Since transcription of *YAK1* is induced by Msn2/4, activation of Msn2/4 can produce more Yak1, generating a positive feedback loop. Therefore, Yak1-dependent activation of Msn2/4 may provide another layer of PKA-dependent regulation of Msn2/4 to ensure proper and sophisticated cellular responses depending on nutrient availability. Beside PKA, Snf1 and Ssn3/Srb10 have been shown to phosphorylate and inhibit Msn2. Snf1 inhibits Msn2 by phosphorylating one of the four PKA sites in NLS during prolonged glucose depletion (De Wever et al., 2005), whereas Ssn3/Srb10 in RNA polymerase II mediator complex is responsible for phosphorylation of Msn2 upon heat shock, which leads to inhibition of Msn2 by degradation (Chi *et al.*, 2001, Lallet *et al.*, 2004, Lallet *et al.*, 2006). In addition, yeast homologues of glycogen synthase kinase-3 (GSK-3) have been shown to increase STRE-binding activity of Msn2 without affecting nuclear localization of Msn2, but via indirect pathway (Hirata *et al.*, 2003). Therefore, Yak1 is the first example shown to activate Msn2 by direct phosphorylation.

Interestingly, it has been shown that Yak1 is also involved in activation of Msn2/4 upon heat shock without affecting heat shock activation of Hsf1. Although it has been well known that Msn2/4 is activated by heat shock, little is known about its activation mechanism. The fact that Yak1 can mediate the heat shock activation of Msn2/4 may contribute to understanding the signal transduction pathways for heat shock activation of Msn2/4. Further research might be necessary to elucidate more comprehensive activation mechanism of Msn2/4 by Yak1-dependent phosphorylation.

Yak1 kinase has long been recognized as a growth antagonist downstream of PKA (Garrett & Broach, 1989). Although it has been shown that Yak1 is onvolved in transcriptional repression of ribosomal protein genes (Martin et al., 2004), that might not be enough to explain the growth inhibition mediated by Yak1. In this study, a central role for Yak1 in coordinating stress responses by activating two key transcription factors, Hsf1 and Msn2/4, under the conditions of low PKA activity was unveiled. Considering that both Yak1 and Msn2/4 are responsible for the growth defect of a PKA deletion mutant, the growth inhibitory effect of Yak1 could be partly related to its regulation of Msn2/4. Such a complex, but sophisticated and efficient regulatory network would ensure proper balance between cell growth and stress adaptation in response to ever changing environmental conditions.

Stress-responsive transcription factors, Hsf1 and Msn2, are activated by Yak1 and Rim15 by different mechanisms

Yak1 and Rim15, common downstream effectors of PKA and TORC1 signaling pathways, play critical roles in proper entry into the quiescent stage upon nutrient depletion through regulation of gene expression. Previously in chapter4, it has been shown that Yak1 activates Hsf1 and Msn2/4 by direct phosphorylation (Lee et al., 2008). Rim15 has been known to function in transcriptional activation of Msn2/4 and Gis1 target genes, but the activation mechanisms have not yet been explored. Although Msn2/4 and Gis1 have redundant functions by sharing many common targets, our study has revealed that Msn2, but not Gis1, is a direct substrate of Rim15. The kinase activity of Rim15 has been shown to be necessary for the Gis1-dependent transcription (Zhang & Oliver, 2010), but Rim15 may contribute to the activation of Gis1 through indirect pathway. Furthermore, it has been shown that Rim15 can activate Hsf1 by direct phosphorylation. Therefore, in response to glucose starvation, Yak1 and Rim15 may work cooperatively by phosphorylation and subsequent activation of common targets Msn2/4 and Hsf1 (Fig. 5.5). However, Yak1 and Rim15 seem to phosphorylate different sites in Msn2 and Hsf1,

implying that although Yak1 and Rim15 have redundant functions, each kinase might exert slightly different level of activation on the transcription factors, providing additive effects on the transcriptional activation.

Rim15 is involved in not only transcriptional activation, but also posttranscriptional stabilization of the induced transcripts via phosphorylating Iog1/2 (Luo et al., 2011). Although the role for Yak1 in posttranscriptional regulation has not yet been demonstrated, Yak1 phosphorylates Pop2, a component of Ccr4-Not deadenylase complex regulating 3' to 5' mRNA decay (Moriya et al., 2001). The Yak1-dependent phosphorylation site in Pop2 has been shown to be necessary for G_1 arrest upon glucose depletion (Moriya et al., 2001). Therefore, it is possible that Yak1 might also regulate mRNA stability of a subset of transcripts through regulation of Pop2 in response to nutrient signals. In summury, in addition to their own specific functions, Yak1 and Rim15 seem to commonly regulate expression of Hsf1 and Msn2/4 target genes by differential phosphorylation of the transcription factors. Such a cooperative and sophisticated regulation of the central stress-responsive transcription factors might ensure proper cellular adaptation and survival in response to a diverse range of nutrient starvation conditions.

Roles of PKA-dependent phosphorylation and Ser/Thr autophosphorylation in regulation of subcellular localization and kinase activity of Yak1

In this thesisdy, molecular mechanisms by which subcellular localization and kinase activity of Yak1 are regulated depending on glucose availability were elucidated. Previous mass spectrometry-based phosphopeptide mapping revealed at least eleven in vivo phosphorylation sites in Yak1, which include five putative PKA sites, four putative CDK sites, and one Tyr autophosphorylation site in the activation loop (Zappacosta et al., 2002). In vitro phosphorylation assays using recombinant GST-Tpk1 have revealed that S295 is the major site phosphorylated by Tpk1, with minor contributions of S127 and S128. Ser to Ala mutations of these three phosphorylation sites, located next to two putative NLSs, led to nuclear localization of Yak1 even under glucose-rich conditions, indicating that PKA-dependent phosphorylation of Yak1 might be the major regulatory mechanism retaining Yak1 in cytoplasm. Yak1 might translocate to the nucleus when the PKA activity is lowered by glucose starvation (Fig. 6.8).

At least three Ser and Thr residues, S234, T288, and S302, out of the four putative CDK sites were turned out to be

autophosphorylation sites of Yak1. Autophosphorylation on one remaining site, S247, could not be verified, because of the instability of GST-Yak1^{S247A} recombinant protein. However, S247 could also be an autophosphorylation site considering the conservation of S/TP motif in all four sites. Additional autophosphorylation site, T335, which is the major autophosphorylation site existing in the context of a putative 14-3-3 binding motif was identified. It has been demonstrated that autophosphorylation occurs via intramolecular phosphorylation event. Furthermore, it has been shown that autophosphorylation on these Ser or Thr residues, which are located in the N-terminal noncatalytic domain, is essential for kinase activity of Yak1. It has been known that DYRKs can autophosphorylate on Ser/Thr as well as Tyr, and Tyr autophosphorylation within the kinase activation loop is essential for kinase activity (Alvarez et al., 2007, Himpel *et al.*, 2001). However, Yak1 is the first example exhibiting a requirement of Ser/Thr autophosphorylation for full kinase activity.

Bmh1 binding to autophosphorylation sites inhibits kinase activity of Yak1

It has been demonstrated that Bmh1 binds to the Ser/Thr autophosphorylation site(s) of Yak1 and inhibits its kinase activity. The major autophosphorylation site of Yak1, Thr335, serves as a primary binding site for Bmh1, but additional binding site(s) in the N-terminal domain seems to be necessary for stable binding of a Bmh1 dimer. It has been shown that each subunit of a 14-3-3 dimer can be engaged in interaction with a binding partner through multiple sites (Obsil *et al.*, 2001, Tzivion *et al.*, 1998, Zha *et al.*, 1996). In many cases, there is a predominant high affinity binding motif called 'gatekeeper', which is involved in the recruitment of a 14-3-3 dimer to the binding partner. Only after this initial binding, another 14-3-3 monomeric subunit can associate with the second low affinity site, stabilizing the interaction (Yaffe, 2002). Mutation of the individual autophosphorylation site of Yak1, except Thr335, did not weaken the interaction with Bmh1 in yeast two-hybrid assays. However, because the Yak1^{T335A} mutant showed slightly higher Bmh1 binding affinity than kinase-inactive Yak1^{K398R} mutant, it is still possible that one or more other autophosphorylated Ser residues can provide the second binding motif for Bmh1.

It has been shown that kinase activity of DYRK1A, one of the mammalian DYRKs, is also regulated by 14-3-3 binding (Kim et al., 2004, Alvarez et al., 2007). Similar to the mechanism of Bmh1 binding

to Yak1, 14-3-3 binds to autophosphorylated Ser520 residue existing near the PEST domain outside of the DYRK1A catalytic domain binding (Alvarez et al., 2007). However, unlike Yak1, autophosphorylation seems not to be directly involved in activation of DYRK1A kinase activity. Instead, it has been shown that binding of 14-3-3 to the phosphorylated Ser520 residue increases DYRK1A kinase activity binding (Alvarez et al., 2007). Therefore, 14-3-3 binding triggers opposite effect on kinase activities of Yak1 and DYRK1A, inhibiting Yak1 while activating DYRK1A.

Based on the observation that Bmh1 binding inhibits kinase activity of Yak1, it was investigated whether Bmh1 binding site are conserved among all eukarytotes. Therefore, full-length homologous protein sequences of Yak1 in diverse eukaryotic organisms were aligned. Interestingly, Bmh1 binding motif, RXXS/TXP, is highly conserved in lower eukarotes (Fig 8.1) including *S. pombe, C. glabrata, C. albicans, K. lactis, P. pastoris, C. neoformans, A. niger, D. discoideum*, but not in higher eukaryotic organisms. However, multiple alignments indicate that PKA phosphorylation sites of Yak1 are not conserved among eukaryotes. Therefore, it would be interesting to test whether the function of Bmh1/2 identified in *S. cerevisiae* is also conserved in other related organisms.

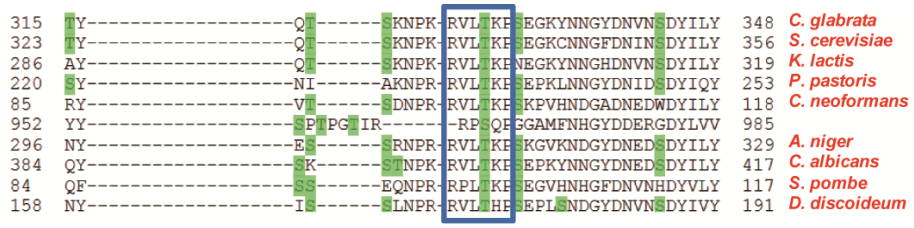

Fig 8.1 Conservation of Bmh1 binding site in multiple alignment of Yak1 homologues. The Bmh1 binding sites are indicated as a box.

As far as we know, Yak1 is the first example showing inactivation of kinase activity by Bmh1 binding in yeast. However, kinase activity of several mammalian protein kinases such as PKCμ, Raf-1, Wee1, Pdk1, CaMKK1, MEKK3 has been shown to be regulated by 14-3-3 binding in both positive and negative ways (Davare *et al.*, 2004, Hausser *et al.*, 1999, Light *et al.*, 2002, Rothblum-Oviatt *et al.*, 2001, Sato *et al.*, 2002, Yip-Schneider *et al.*, 2000). 14-3-3 binds to phosphorylated residues either in catalytic or noncatalytic domain, generated by either autophosphorylation or phosphorylation via upstream kinases. PKCμ shows similar 14-3-3-dependent regulation pattern to that of Yak1 in that 14-3-3 binds to autophosphorylation site of PKCμ and inhibits its kinase activity (Hausser et al., 1999).

Based on the observation that Bmh1/2 bind to Yak1 only in the presence of glucose, concomitant with cytoplasmic localization of Yak1, it has been suggested that Bmh1/2 might be responsible for nuclear exclusion of Yak1 (Moriya et al., 2001). However, a Yak1^{K398R} mutant, which cannot bind to Bmh1/2, showed normal nucleocytoplasmic shuttling depending on glucose availability, suggesting that Bmh1/2 binding is not essential for regulation of subcellular localization of Yak1. Moreover, PKA-dependent phosphorylation, which is essential for cytoplasmic retention of Yak1, does not affect Bmh1 binding. It is not clear yet how Bmh1/2 binding to Yak1 is regulated in a glucose-

dependent manner. Since most 14-3-3 binding requires phosphorylation sites, condition-dependent regulation of kinase or phosphatase activity has been reported as a major mechanism regulating 14-3-3 binding. However, Bmh1 binds to Yak1 autophosphorylation site(s) required for kinase activity. Since Yak1 is active under the conditions of abolished Bmh1/2 binding, it is unlikely that the release of Bmh1/2 from Yak1 involves dephosphorylation of the autophosphorylation site, which could result in inactivation of Yak1. Therefore, Bmh1/2 release upon glucose starvation could possibly be triggered by phosphorylation on other residues within the binding motif as demonstrated for regulation of 14-3-3 binding to p53 and Cdc25 (Bulavin *et al.*, 2003, Waterman *et al.*, 1998). Otherwise, Bmh1 or Bmh2 itself could possibly be a target for regulation. Although it has not been reported for Bmh1/2, the activity of 14-3-3 in higher eukaryotes has been shown to be regulated by phosphorylation or binding of ligands such as cAMP and Ca^{2+}.

In summury, this study has revealed a novel Ser/Thr autophosphorylation-dependent activation mechanism of Yak1 and its regulation of nucleocytoplasmic shuttling and kinase activity by PKA and Bmh1/2, respectively (Fig. 6.8). PKA-dependent phosphorylation together with Bmh1/2 binding results in cytoplasmic retention of inactive Yak1 in the presence of glucose. Whereas, glucose starvation

triggers inactivation of PKA and release of Bmh1/2 from Yak1 by a yet unidentified mechanism, leading to activation of Yak1 in the nucleus where target proteins such as Hsf1, Msn2/4, and Crf1 are located. It is interesting to note that such regulatory roles of PKA and Bmh1/2 are apposite for the regulation of Rim15 kinase. It has been shown that PKA-dependent phosphorylation inhibits kinase activity of Rim15, without affecting its subcellular localization, whereas 14-3-3 binding is responsible for cytoplasmic retention of Rim15 (Wanke et al., 2005, Reinders et al., 1998, Wanke *et al.*, 2008). Sch9, which is activated by TOR, and Pho85, a phosphate-sensing CDK, have been shown to phosphorylate Rim15 on Ser1061 and Thr1075, respectively, providing 14-3-3 binding sites for cytoplasmic retention (Wanke et al., 2008, Wanke et al., 2005). TOR also has been shown to contribute phosphorylation on Thr1075 via inactivation of a yet unidentified phosphatase (Wanke et al., 2005). Therefore, nutrient-sensing kinases and 14-3-3 proteins seem to adapt various target-specific regulatory mechanisms to coordinate cellular processes in response to nutrient availability.

Potential biotechnological applications

S. cerevisiae has served as a very useful model organism to understand fundamental biological processes that are conserved from yeast to humans. Because it is a safe organism with readily available genetic manipulation tools, *S. cerevisiae* is also considered as a valuable system in a wide range of biotechnological applications. In addition to its popular usages in the traditional food and beverage industries, *S. cerevisiae* is a promising host to produce various useful chemicals as well as ethanol. Understanding the yeast cell growth and stress responses during the fermentation is essential to improve the productivity of target chemicals. In this thesis, the regulation and function of Yak1 as well as Rim15 in response to nutrient conditions was elucidated in *S. cerevisiae*. Yak1 and Rim15 kinases may have evolved to integrate nutrient signals transduced via nutrient sensing kinases such as PKA and TOR, to appropriately regulate the stress responses and growth arrest that ensure cell growth under favorable conditions and cell survival under less favorable conditions. Therefore, the newly elucidated signal transduction pathways would provide useful information to engineer yeast cells for biotechnological applications.

Furthermore, it has also been shown that Yak1 is involved in hyphal and adhesive growth (Goyard et al., 2008, Iraqui et al., 2005). Especially for some pathogenic Candida species, biofilm formation is highly related to their pathogenicity. Therefore, if the regulatory mechanisms for Yak1 activity are also conserved in those organisms, the newly identified regulatory pathways might provide potential targets for biofilm control.

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초 록

출아성 효모인 *Saccharmocyes cerevisiae* 에서 이중 특이성 타이로신 인산화 조절 키나아제로 알려진 Yak1 은 진화적으로 잘 보전된 Ser/Thr 인산화 효소이며, Ras/PKA 신호전달 경로의 하위에서 그 기능을 하는 성장 억제자로 알려져있다. *In vitro* 실험조건에서 PKA 에 의해 인산화되며, 영양분 고갈에 따라 핵으로 이동됨이 알려져 있지만, Yak1 의 타겟과 Yak1 의 활성 및 위치에 대한 조절 메커니즘에 대한 연구는 미흡하다. 본 연구에서 Yak1 은 새롭게 찾아진 타겟 전사조절인자들인 Hsf1 과 Msn2/4 를 포도당 고갈 상태에서 활성화하는 기능이 규명되었으며, PKA 와 Bmh1/2 를 매개로 한 Yak1 의 새로운 조절 기전 또한 규명되었다.

첫번째로 포도당 고갈 또는 세포내 cAMP 를 가수분해하는 Pde2 를 과발현하여 PKA 의 활성을 저해하였을 때, Hsf1 과 Msn2 가 Yak1 인산화 효소에 의해 활성화됨이 규명되었다. Hsf1 과 Msn2/Msn4 는 열충격, 산화적 스트레스 및 영양분 고갈 같은 다양한 스트레스에 대한 유전자 발현의 활성을 조절함으로써 세포의 항상성에 중요한 역할을 담당한다. 그리고 *In vitro* 실험에서

Hsf1 의 DNA 결합력은 Yak1 에 의한 인산화에 의해 증가됨이 확인되었다. 이와는 달리 Msn2 의 경우, DNA 결합력 또는 세포 내 위치의 이동은 Yak1 에 의한 인산화 여부에 영향을 받지 않음이 확인되었다.

두 번째로 PKA 와 TORC1 에 의해 억제 조절을 받고 있는 Yak1 뿐만 아니라 Rim15 은 전사 활성과 전사물들의 안정성을 통해 포도당 고갈에 따른 Hsf1 의 타겟 유전자들의 발현을 촉진한다. *In vitro* 조건에서 Hsf1 이 Rim15 에 의해 인산화되는 결과는 Rim15 이 Hsf1 을 직접적으로 활성화할 수 있음을 시사한다. 그리고 Rim15 에 의해 인산화되는 Igo1 과 Igo2 가 Hsf1 의 타깃 유전자인 BTN2 mRNA 을 보호할 수 있음이 확인되었다. 추가적으로 Rim15 이 Gis1 을 제외한 Msn2 만을 인산화함이 확인되었다. 이러한 결과는 Msn2 와 Gis1 이 Rim15 에 의해 서로 다른 기작으로 활성화됨을 의미한다.

세번째로 Yak1 의 조절에 있어 PKA 와 효모에서 14-3-3 단백질로 알려진 Bmh1 의 역할을 조사하였다. PKA 에 의한 Yak1 의 인산화 위치를 증명하였으며, 이를 통해 Yak1 의 세포 내 위치가 PKA 에 의해 조절됨을 규명하였다. 그리고 Yak1 의 최대 인산화효소 활성에 아미노 말단에 존재하는 네 곳의 Ser/Thr

자가인산화가 필요함을 규명하였다. 추가적으로 아미노 말단에 Bmh1 의 결합 위치인 335 번째 Threonine 을 규명하였으며, Bmh1 의 결합에 의해 Yak1 의 세포 내 위치이동에는 영향이 없으나 인산화효소의 활성이 감소됨을 확인하였다. Bmh1 이 Yak1 에 결합됨과 PKA 의 활성기작이 동시에 일어나는 조절 메커니즘을 고려할 때, 포도당의 조건에 따라 Yak1 의 효소활성과 세포 내 위치가 협조된 조절을 받을 것으로 판단된다.

종합적으로 본 연구는 영양분 고갈 상태에서 Yak1 과 Rim15 이 세포 내 스트레스 반응과 성장을 억제시키는 것을 촉진함으로서 세포의 생존을 유지하도록 하는데 중요한 역할을 하고 있음을 시사한다.

주요어: 자가인산화, Bmh1/2, Hsf1, Msn2/4, 영양소 고갈, 인산화, PKA, Rim15, TOR, Yak1

학 번: 2006-21369

감사의 글

2006년 2월에 시작된 실험실 생활이 엊그제 같은데 이제 한 분야의 전문가로써 졸업을 하게되었습니다. 그 동안 많은 일들이 생물분자공학실험실에서 있었습니다. 그 많은 시간과 과정 동안 느껴왔던 감사함을 이곳에 담고자 합니다.

먼저 실험실에 처음 들어와 기초 실험부터 여러 방면에서 저의 부족함을 도와주시고 지도해주신 한지숙 교수님. 학위 과정 동안 교수님께 정말 많이 배웠습니다. 교수님의 제자로써 배움 하나하나를 기억하며 그에 걸맞은 능력을 키워 나가는 과학자가 되도록 노력하며 살겠습니다. 진심으로 감사 드립니다. 그리고 박사 학위 심사위원을 맡아주신 김병기 교수님, 박태현 교수님, 허원기 교수님, 그리고 반용선 교수님. 바쁘신 일정에도 많은 지도와 격려를 주셔서 감사 드립니다.

지난 학위과정 동안 동고동락하며 지내왔던 저희 생물분자공학 실험실 멤버들에게 감사의 마음을 전합니다. 윤정누나, 명혜누나, 태준이형, 보람형, 규성이, 상규, 순화, 강현이, 수진이, 승호, 아람이, 상민이, 대희, 명섭이, 보수, 성희. 앞으로 실험실 더 잘 이끌어 나가길 바랄게요.

실험실 생활을 하며 알게 되고 연구에 관한 이야기뿐만 아니라 서로를 격려 해주며 지내온 분들께도 감사의 마음을 전합니다. 생물방이라 불리는 ‘ 효소 및 환경생물공학 연구실, 분자생물공학 및 생물 신소재연구실, 세포 및 미생물공학 연구실, 단백질 신소재 연구실 ’ 그리고 자연대 노정혜 교수님 실험실, 허원기 교수님 실험실의 선후배분들께 감사의 마음을 전합니다.

한신교회 목사님과 성도들에게도 감사의 마음을 전합니다. 담임목사님이신 강용규 목사님. 목사님께서 말씀해 주신 ‘믿음, 소망, 사랑’ 안에서 살아가겠습니다. 그리고 김요섭 목사님, 정승훈 전도사님, 한신 청년부 친구들, 금요철야예배 찬양팀인 디아코니아 멤버들에게도 감사의 마음을 전합니다. 주님 안에서 아름다운 가정을 이루며 살겠습니다.

대학원 룸메였던 용범이형, 많은 추억을 함께한 현준이형, 승열이형, 종혁기형, 일본에 있는 영빈이형. 친구들 빛내리, 형관이, 한영이, 재욱이, 세창이, 너희들의 아낌없는 조언 너무 고맙다.

마지막으로 저희 가족에게 감사의 마음을 전하고 싶습니다. 처음부터 끝까지 저를 믿어주시고 힘을 주시는 아버지, 어머니 그리고 항상 응원해주시고 격려해주시는 장인 어른, 장모님. 누나들과 매형들, 도연 처형, 도희 처제. 진심으로 감사하고 사랑합니다. 또한 박사학위를 받는 과정중에 응원해주고 격려해주고 기뻐해준 나의 아내 도경이에게 사랑한다고 고맙다고 전하고 싶습니다. “도경아~결혼 준비하느라 대학원 졸업 준비하느라 그리고 오빠 챙기느라 정말 고생했어.”

끝으로 학위 과정 동안 부족함을 채워주시고 능력을 허락해주신 하나님의 한량없는 은혜에 감사 드립니다.

2012 년 8 월

관악에서 감사의 마음을 담아… 베드로 올림