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The regulation of stress induced genes by yeast transcription factor *GCN4*

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Yeast cells respond to condition of amino acid starvation by synthesizing *GCN4*, a typical eukaryotic transcriptional activator, which regulates the expression of many amino acids biosynthetic genes. By introducing point mutations in the DNA binding domain of *GCN4*, mutants with normal DNA binding activity but defective in transcriptional activity were isolated to identify unknown proteins that could suppress the mutant phenotype under an amino acid depletion condition. As a result, *SSB*(Stress-Seventy B) subfamily proteins were identified as suppressors of mutant *GCN4*. *SSB* proteins were known as a member of yeast hsp70 family that probably aids passage of nascent chain through ribosomes. Among them, the mechanism of suppression by *SSB2* on the defective *GCN4* mutant strains is under investigation. Gcn4p directly interacts with Ssb2p through the basic DNA binding domain of *GCN4*. It suggests the possibility that physical interaction might induce the transcriptional activation of Gcn4p.

Key words : Transcriptional activation, *GCN4*, *SSB*, amino acid starvation

INTRODUCTION

Yeast cells respond to starvation for any single amino acid by coordinately activating at least 40 genes in amino acid biosynthetic pathway through the activation of Gcn4p, a typical eukaryotic transcription activator (Hinnebusch A. G., 1988). Gcn4p is a member of bZIP family transcription factors which contain a basic region-leucine zipper (bZIP) DNA binding domain. These protein include c-Jun, c-Fos, CREB, c-Myc, yAP-1 and so on.

Gcn4p is known as a yeast functional homolog of a c-Jun human oncoprotein (Hill, D.E. *et al.*, 1986) and binds directly as a homodimer (Hope, I.A. *et al.*, 1987) to a conserved regulatory sequence 'TGA(C/G)TCA' of its target genes (Hill, D.E. *et al.*, 1986). This sequence is the same as a target sequence of mammalian AP-1 family transcription factors (Struhl K., 1987). *GCN4* expression and activity are subject to a tight series of controls that are exerted at the transcriptional (Albrecht, G. *et al.*, 1998), translational (Mueller and Hinnebusch, 1986) and post-translational levels (Kornitzer, D. *et al.*, 1994). Gcn4p has an N-terminal ~125 amino acid activation domain containing seven clusters of hydrophobic amino acids (Jacson B. M., 1996) that mediate interactions between the activation domain (Drysdale M. *et al.*, 1995) and various co-activator proteins in cell extracts, including components of SAGA complex (Natarajan K. *et al.*, 1998), RNA polymerase II holoenzyme mediators and TFIID (Drysdale M. *et al.*, 1998). However, there are reports that a DNA-binding domain was also related to transcriptional activation in some other transcription activators. (Ha N. *et al.*, 1996, Kim, K. *et al.*, 1989). Therefore, it might be possible that the transcriptional activators including bZIP family proteins can be expected to have multiple functions in their DNA binding domains.

In this study, Gcn4p mutants that have a normal DNA-binding activity with point mutations in its DNA-binding domain but a defect in transcription activation were isolated to identify unknown proteins that could suppress the mutant phenotype under an amino acid starvation. We found the *SSB2* (Stress-Seventy subfamily B2) gene as a suppressor.

Ssb2p is a member of yeast HSP70s (Craig, E. A. and Jacobson K., 1985, Craig, E. A. *et al.* 1995), which are conserved molecular chaperones that participate in a variety of cellular functions, including protein folding, transport and the repair of stress-induced damage (Morimoto, R. I. *et al.*, 1994, Stone, D. E. *et al.*, 1990, Halladay, J.*et al*, 1995). Ssb2p is associated with translating ribosomes and can be cross-linked to nascent polypeptides preventing the misfolding of newly synthesized proteins (Nelson, R. J. *et al.*, 1992, Pfund, C., 1998). The suppression by Ssb2p for the mutant proteins is not known yet. We present here the first lines of evidence that Ssb2p suppresses the defective Gcn4p phenotype by direct binding.

We also showed that the direct interaction between Gcn4p and Ssb2p seems to be associated with the increase of the target gene expression. These data demonstrate the

gcn4 mutants with defective activity may be compensated with overexpressed Ssb2p.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions. *Saccharomyces cerevisiae* strains used in this study are KY321 (MATa *trp1-Δ1 ura3-52 leu2 lys2-801* gcn4::HIS3) and KY803 (MATα *trp1-Δ1 ura3-52 leu-P1 gcn4-Δ1*). Transformations were carried out using lithium acetate yeast transformation method (Ito, H. *et al.*, 1983) and these transformants were spread on SC plate without appropriate amino acids. For amino acid starvation, 3- amino-1,2,4-triazole (3-AT) was added to cultures grown to mid-log phase.

Plasmids and Constructs *GCN4* derivatives were cloned into a low copy plasmid vector pRS315 with *LEU2* selectable marker for suppression test. *SSB2* was cloned into a high copy plasmid vector YEp24 with *URA3* selectable marker. *GCN4* deletion mutants were made by PCR amplification. For GST-pull down assay, Glutathione-S-transferase (GST) was fused to the N-terminal of *GCN4* derivatives in pGEX-5X-1 and 6× Histidine(His)-tagged *SSB2* was cloned into pET21a.

RNA Preparation and Analysis Total RNA was isolated from yeast cultures grown in SC-HUL media to an optical density at 600nm of 0.6~0.8 by Hot-phenol method. For Northern hybridization, 25㎍ total RNA per lane was separated on a formaldehyde agarose gel and transferred onto nylon membrane by capillary transfer. This was hybridized with DIG-labeled gene-specific probe and detected by using the Chemiluminescent Detection Kit(CDP-SATR™, Boeringer Mannheim Inc. Cat. No. 1685627).

In Vitro Binding Assay and Western Blotting GST fused GCN4 derivatives and His-SSB2 were overexpressed in E. *coli* BL21pLysS. After GST--GCN4 derivatives were bound to glutathione sepharose 4B (Amersham Pharmacia Biotech Inc.) beads, His-SSB2 overexpressed cell lysates were loaded and incubated with rotation for overnight at 4℃ in binding buffer which is composed of 40mM HEPES(pH 7.6), 20% glycerol, 1mM DTT, 5mM ATP, 0.3 μM phenylmethyllsulfonyl fluoride and protease inhibitor mixture (pepstatin A, leupeptin, aprotinin ; 5μg/㎖ each). The reaction mixture was then washed three times with binding buffer containing 0.1% Triton X-100 and GST, GST-*GCN4* derivatives were eluted with 15mM reduced glutathaione. The eluted proteins were separated by 12% SDS polyacrylamide gel electrophoresis. Then, each proteins was transferred to nitrocellulose membrane by electroblotting. The Western blotting was achieved using primary rabbit anti-GST monoclonal antibody and mouse anti-His monoclonal antibody (Santa Cruz Biotechnology Inc.) and secondary peroxidase conjugated monocloanal goat anti-rabbit IgG and goat anti-mouse IgG (Sigma), respectively. The blot was subjected to Western analysis using chemiluminescence system (Boeringer Mannheim Inc.).

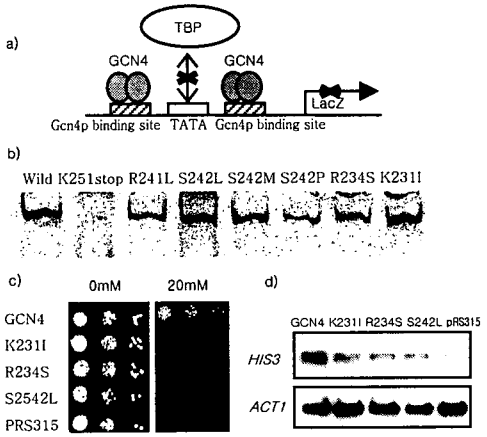

Figure 1. Isolation of gcn4 mutants. a) YCp87-JK3. GCN4 DNA-binding sites were inserted to both sides of TATA element . Binding of GCN4 derivatives results in inhibition of LacZ expression due to steric hindrance. b) In vitro translated Gcn4p mutants, labeled ^{35}S-methionine, were used for EMSA. Showing band is a DNA-protein complex. c) Transcriptional activity of mutant Gcn4p was tested by using 3-aminotriazole. Cell suspension was serially diluted and spotted on SC-HL plate. d) KY321a strain was transformed with the indicated constructs and the expression levels of *HIS3* and *ACT1* were detected by northern hybridization analyses as described in Materials and Methods.

S1 nuclease analyses Total RNA was isolated by a hot-phenol method from yeast cells grown in synthetic complete medium lacking histidine, leucine and uracil and then treated with 30mM aminotriazole (AT) for 6hr. Portions of the RNA (100㎍) were subjected to S1 nuclease analyses with ^{32}P-labeled oligonucleotide probes for *HIS3* and *DED1* genes as described previously(Iyer, V. et al., 1996)

RESULTS

***GCN4* mutants isolation by saturation mutagenesis** Gcn4p is a master regulator which is synthesized during amino acid starvation condition and induce the transcription of amino acid biosynthetic genes. (Hinnebusch A. G., 1988) To test whether the DNA-binding domain of Gcn4p also has a transcriptional activation activity, gcn4 mutants that have point mutations in the DNA-binding domains but have the normal DNA-binding activities, were isolated by saturation mutagenesis with spiked oligonucleotides in the DNA binding domain. We confirmed the normal DNA-binding activity of gcn4 mutants through X-gal test and EMSA and also checked the decreased transcriptional activity by using 3-AT, a competitive inhibitor of *HIS3*, and northern hybridization. Although these mutants have a normal DNA binding activities, they have defective transcriptional activation

activities(Fig.1). Especially, K231I, R234S, S242L mutants have good DNA-binding activities and decreased transcriptional activities.

Overexpressed *SSB2* suppresses the defective phenotype of *GCN4* mutant strains. To identify the unknown protein that overcome the decreased transcriptional activity of *gcn4* mutant, yeast genomic library was used and this genomic library was carried in a high copy plasmid to raise the possibility for the interaction between *gcn4* mutant and unknown protein. As a result, we found the clone that could grow under amino acid depletion condition and this gene was identified as *SSB2* (Stress-Seventy subfamily B2) by sequence analysis. To confirm the *SSB2* with respect to the suppression, we evaluated the ability of *GCN4* mutants to confer resistance to 3-amino-1,2,4-triazole (3-AT) which is the competitive inhibition of IGPD and induced the histidine depletion condition (Ito, H. *et al.* 1983)(Fig.2). KY321a strain lacking *GCN4* was transformed with a low copy plasmid vector expressing each *GCN4* derivatives and a high copy plasmid vector expressing *SSB2*. Because *GCN4* gene promoter region was replaced with the constitutively expressing promoter of *DED1*, this experimental system was not regulated by the translational control. Wild type *GCN4* grows on media containing 3-AT without *SSB2*. However, each *gcn4* mutants ought to have the *SSB2* to survive on the 3-AT media. These data indicate that the *SSB2* might have an important role as a suppressor for the defective *GCN4* in cell survival under an amino acid starvation condition.

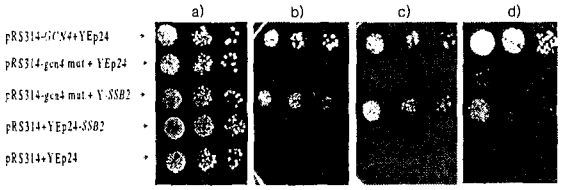

Figure 2. Suppression for each *gcn4* mutant with *SSB2* (a) 0mM AT (control) (b) K231I *gcn4* mutant (c) R234S *gcn4* mutant (d) S242L *gcn4* mutant. (b), (c) and (d) 20mM AT each. A strain lacking the chromosomal *GCN4* gene was co-transformed with the two different plasmids: the vector plasmids (pRS314, YEp24), the each vector carrying *GCN4* derivatives and *SSB2* as indicated above. Appropriate transformants were evaluated for their ability of growing on SC-HUW media, SC-HUW plus 20mM 3-AT. To be equal to each other in cells number, transformants were grown to an optical density at 600nm of 0.6 and serially diluted. Spotting on 0mM AT media for each *gcn4* mutants was performed, but the only one was shown.

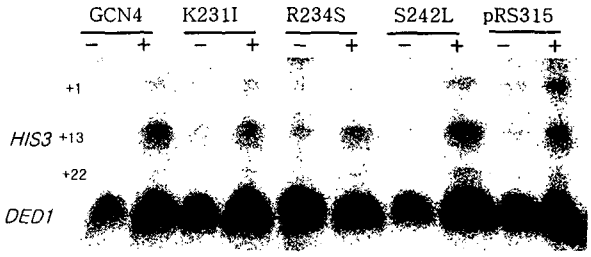

Figure 3. Effect on *HIS3* transcription. Total RNA from each transformants harboring the indicated plasmids was subjected to S1 nuclease analysis. (- :YEp24, +:YEp24-SSB2)

Expression of *SSB2* is Gcn4p-dependent. In addition, if these *gcn4* mutants with normal DNA binding activities are suppressed by *SSB2*, it is possible that *SSB2* may help the *GCN4*-dependent transcription. To identify this idea, mRNA levels of *HIS3*, major target genes of *GCN4*, were evaluated. Under histidine starvation, decreased mRNA level of *GCN4* target genes were restored when *SSB2* was introduced on the *gcn4* mutant strains. Transcription of *HIS3* occurs at the two initiation sites (+1, +13) and *GCN4* activates *HIS3* transcription through the +13, but not the +1 under amino acid starvation condition. To investigate further, we examined the *HIS3* mRNA level by S1 nuclease mapping (Fig. 3). These data indicate that Ssb2p might help defective Gcn4p by stimulating the gene expression under the amino acid starvation condition.

***SSB2* directly interacts with *GCN4*.** Gcn4p stimulates a lot of genes under an amino acid starvation condition through the interaction with other proteins including SAGA complex (Natarajan, K. *et al.* 1998), SRB mediators of RNA Pol.II and TFIID (Drysdale, C. M. *et al.*, 1998) and a high copy cross suppression is known as a powerful method to study a specific macromolecular interaction. Therefore, we thought that this interaction might affect the suppression of *SSB2*. To identify whether Gcn4p interacts with Ssb2p, GST-pull down assay was performed. Surprisingly, the suppressor *SSB2* bound both wild type *GCN4* and defective *gcn4* mutant. The strength of the protein-protein interaction might be an important clue in estimating their function. To investigate the difference of the affinity between wild type *GCN4* and defective *gcn4* mutant, the salt concentration in the binding buffer was enhanced (Fig. 4). Although both bindings became significantly weaker, the binding affinity with wild type *GCN4* was stronger than that of defective mutant. This means that Ssb2p, if necessary, could interact with wild type Gcn4p and function operate according to the surrounding conditions for survival.

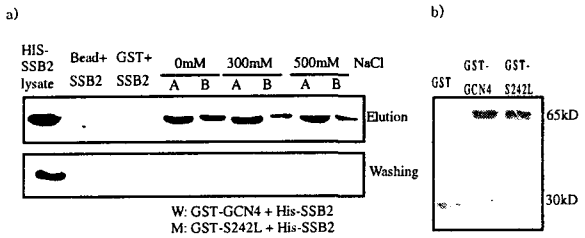

Figure 4. Comparison of binding affinity between wild *GCN4* and S242L *gcn4* mutant. (a) Interaction of *SSB2* with *GCN4* derivatives in *in vitro* binding assay as descrived in Materals and Methods. The binding buffer contained indicated NaCl concentrations indicated at the top. Bound protein was detected using an anti-His antibody. (b) Gel was stained with Coomassie brilliant blue to show equal load of GST fusion proteins. 1: GST(alone), 2: GST-*GCN4*, 3: GST-S242L *gcn4*

Interaction of *SSB2* with *GCN4* requires the basic region of DNA-binding domain of *GCN4*. Next, to determine whether Ssb2p interacts with the bZIP DNA-binding domain of Gcn4p, located between amino acids 221 and 281, as would expected, the deletion mutants were prepared by PCR amplification. *In vitro* binding assay was performed with these mutants as described in materials and methods. Ssb2p interacted with all the constructs except for gcn4 Δ4(a.a 247-284) although Ssb2p had the difference in affinity, this difference may be resulted from conformation change due to the deletion. These data suggested that *SSB2* interacts with basic region in the bZIP DNA binding domain of Gcn4p (Fig. 5).

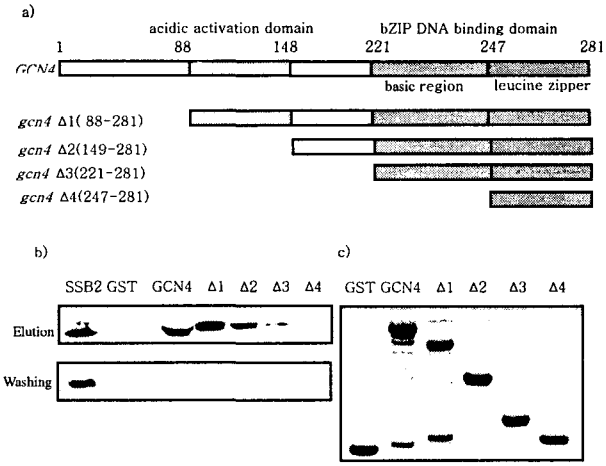

Figure 5. *GCN4* binding domain in interaction with *SSB2* (a) Schematic representation of the *GCN4* derivatives used. (b) In vitro interaction of *SSB2* with *GCN4* requires the basic region in the bZIP DNA binding domain of *GCN4*. Western blotting was performed with anti-His antibody (c) Gel stained with Coomassie brilliant blue was used to show equal load of *GCN4* derivatives

DISCUSSION

Gcn4p is one of the well characterized activators in yeast. It is known that Gcn4p recruits various transcription factors through its activation domain (Natarajan, K. *et al.*, 1998). However, in recent papers, DNA binding domain might have another function in transcriptional activation of a target gene (Ha, N. *et al.*, 1996). The coactivator multiprotein bridging factor(MBF1) mediates Gcn4p - dependent transcriptional activation by bridging the DNA binding region of Gcn4p and TBP(TATA binding protein) (Takemaru, K. *et al.*, 1998). In this study, we isolated the DNA-binding domain mutated gcn4 mutants, which has normal DNA-binding activity but defective transcriptional activity, to verify another function in DNA-binding domain of transcriptional activator. Using these mutants, we found the suppressor, Ssb2p that could overcome the defective transcription through the high copy suppression method(Rose M. D. *et al.*, 1991). And we showed that the Ssb2p might help mutants gcn4 to increase the expression levels of target genes. We thought that this may be acquired through the interaction between the Gcn4p and Ssb2p. Here, we suggest a model based on our data that Ssb2p might influence a Gcn4p mediated transcriptional activation under amino acid deficient condition through the guiding the proper folding of defective gcn4 mutants or stimulating the nuclear localization of those to express target gene for surviving. However, to confirm this mechanism, further study on this will be followed.

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