Protection of Metal Stress in *Saccharomyces cerevisiae*: Cadmium Tolerance Requires the Presence of Two ATP-Binding Domains of Hsp104 Protein

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Received February 20, 2001

We have explored the importance of two ATP binding domains of Hsp104 protein in protection of yeast cells from cadmium exposure. In the previous study we have discovered that the presence of two ATP binding sites was essential in providing heat shock protection as well as rescuing cells from oxidative stress. In this paper we first report wild type cell with functional hsp104 gene is more resistant to cadmium stress than hsp104-deleted mutant cell, judging from decrease in survival rates as a result of cadmium exposure. In order to demonstrate functional role of two ATP binding sites in cadmium defense, we have transformed both wild type (SP1) and hyperactivated *ras* mutant (IR2.5) strains with several plasmids differing in the presence of ATP binding sites. When an extra copy of functional hsp104 gene with both ATP binding sites was overexpressed with GPD-promoter, cells showed increased survival rate against cadmium stress than mutants with ATP binding sites changed. The degree of protection in the presence of two ATP binding sites was similarly observed in *ira2*-deleted hyperactivated *ras* mutant, which was more sensitive to oxidative stress than wild type cell. We have concluded that the greater sensitivity to cadmium stress in the absence of two ATP binding sites is attributed to the higher concentration of reactive oxygen species (ROS) produced by cadmium exposure based on the fluorescence tests. These findings, taken all together, imply that the mechanism by which cadmium put forth toxic effects may be closely associated with the oxidative stress, which is regulated independently of the Ras-cAMP pathway. Our study provides a better understanding of cadmium defense itself and cross-talks between oxidative stress and metal stress, which can be applied to control human diseases due to similar toxic environments.

Keywords : Cadmium stress, Oxidative stress, ATP binding sites.

Introduction

Heavy metal ions often damage viable cells severely even if they play essential roles on many metabolic processes at low concentrations.[1] Molecular mechanisms by which cells defend themselves from metal stresses have been recently studied in yeast because whole DNA sequence is now available and manipulation of yeast genome is rather convenient.

Metal resistance in *Saccaromyces cerevisiae* has been observed *via* several yeast transcription factors, such as CAD1[2] and Yap1.[3] The carboxy-terminal domain of Yap1 was found to act negatively in cadmium resistance.[4] Yap1 has been also induced by oxidative stress and regulate TRX2, which encodes one of the two thioredoxins produced in yeast.[5] Like Yap1, Skn7 is another transcription factor which is important for resistance to H_2O_2. But only Yap1 is important for cadmium resistance, whereas Skn7 has a negative effect upon this response.[6] This difference implies the dissociated function of Yap1 and Skn7 in H_2O_2 and cadmium resistance.

Nothing has been clearly demonstrated to explain how cadmium exert its toxicity on viable cells except that it reacts with thiol groups and can substitute for zinc in certain proteins.[7] In view of defending cells from the diverse types of harmful stresses, heat shock proteins have been intensively investigated in general.[8] A protective role through hsp60 induction,[9] hsp70 and GRP78,[10] and HSP90, HSP72 and HSP27[11] were recently confirmed in cadmium treated cells. It has been suggested that HSP induction by cadmium is due to oxidative stress which mainly involves formation of ROS and more damaged proteins as a signal for induction of heat shock proteins.

Among them, Hsp104 protein has drawn a great attention because it helps yeast cells survive short exposures to both extreme temperatures and severe oxidative damage.[12] In our previous study, the hyperactivated *ras* mutant with *ira2* gene deleted exhibited more sensitive response to several oxidants when Hsp104 protein was overexpressed.[13] A mild heat pretreatment resulted in cross protection between heat shock and oxidative stress.

It is worth noting that Hsp104 protein is highly homologous to the *E. coli* ClipA/ClipB family, which mostly contain the two ATP binding domains and exhibit ATPase activities.[14] When any of two ATP binding sites is altered ($Lys^{218} \rightarrow Thr^{218}$, $Lys^{620} \rightarrow Thr^{620}$, or both), cells lose thermotolerance provided by mild heat pretreatment as well as its ability of resolubilizing aggregated proteins *in vitro*.[15] We have recently observed similar functions of the two ATP binding sites in rescuing cells from oxidative stress as well (manuscript in preparation).

In order to address protecting role of Hsp104 protein in heavy metal stress associated with oxidative stress, both wild type strain and Δ104 mutant were examined not only

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for their survivals upon cadmium exposure but also for their ROS productions. More interestingly, the importance of two ATP binding sites in protection of cadmium stress was addressed using recombinant strains of SP1 and IR2.5 with the plasmid pYS104 (wild type Hsp104), pRS316 (no Hsp104), pKT620 (2nd ATP-binding site mutated), and pKT218/620 (both ATP-binding sites mutated). Finally cadmium defense mechanism was compared with protection of oxidative stress based on our earlier finding that overexpression of functional Hsp104 protein with two ATP binding sites also mediates oxidative stress exerted by several oxidants, such as H_2O_2 and menadione.

Results and Discussion

Cell Survivals upon Cadmium Exposure. In order to explore a protective role of Hsp104 protein against heavy metal stress, cells were incubated with several heavy metal ions, including Cd^{2+}, Cr^{2+}, Hg^{+}, Fe^{2+}, Cu^{2+}, and Ni^{2+}. However their protection patterns were observed only with a limited range of toxic metal ions. In the frame of our study, cadmium was the only one to exhibit significant protection phenotype between strains with different levels of Hsp104 protein expressed. In a manner analogous to heat shock protection, the effect of Hsp104 protein on cell survivals upon cadmium exposure, examined by plating assays, is reported in Figure 1A. Either wild type cell or hsp104 deleted mutant was incubated with various concentrations of cadmium for up to 60 min and stamped on YPD plates with serial dilutions. It appears that wild type cell, containing Hsp104 protein induced by acetate at normal temperature, did not experience the toxic effect of cadmium exposure, if any. On the other hand, cells could not survive even after 30 min exposure at 5mM cadmium chloride in the absence of Hsp104 protein expressed. Measurements of cell survivals under our conditions were also performed by colony counting assays as presented in Figure 1B. It was in a good agreement with Figure 1A, in which wild type cell obtained improved resistance against cadmium exposure whereas the hsp104 deleted mutant showed dramatic decrease of survival rates in a dose dependent manner. These findings confirm that the presence of Hsp104 protein plays crucial role on conferring resistance to cadmium stress.

Generation of ROS upon Cadmium Stress. A protective role through various heat shock proteins has been studied in several cadmium treated cells. It has been suggested that HSP induction by cadmium is due to the oxidative stress mainly consisting in formation of reactive oxygen species and more damaged proteins. In order to verify whether cadmium resistance provided by Hsp104 protein is related to protection of oxidative stress, production of ROS was measured by fluorescence of 2',7'-dichlorofluorosin diacetate

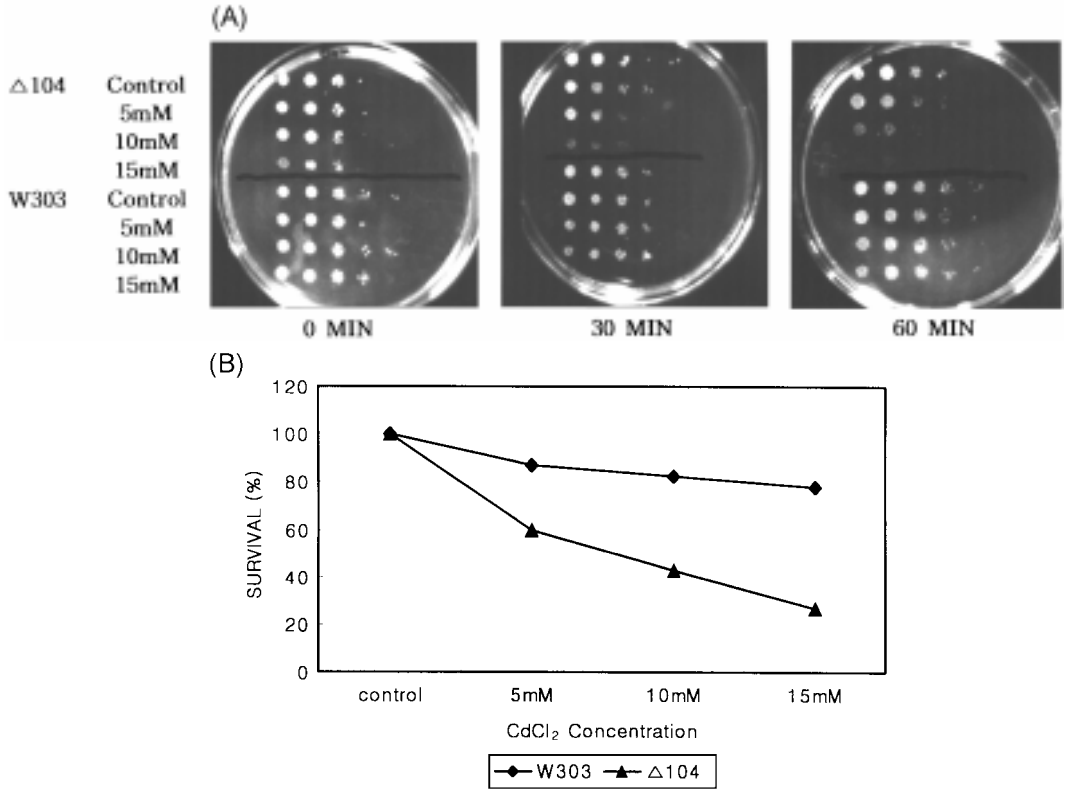

Figure 1. (A) Sensitivity of wild type (W303) and mutant (Δ104) yeast cells upon time-dependent exposure to various concentrations of cadmium. Cells were cultured in acetate to induce HSP104 protein expression and incubated with cadmium. Cells were then diluted in a 96 well plate, stamped onto YPD plates and cultured for 2 days. (B) Measurement of cell survivals by colony-counting assays. After treatment for 30 min at various concentrations, cells were diluted and spread on YPD plates. After 2 days, cells were counted and the survival rates were reported as a percentage of control.

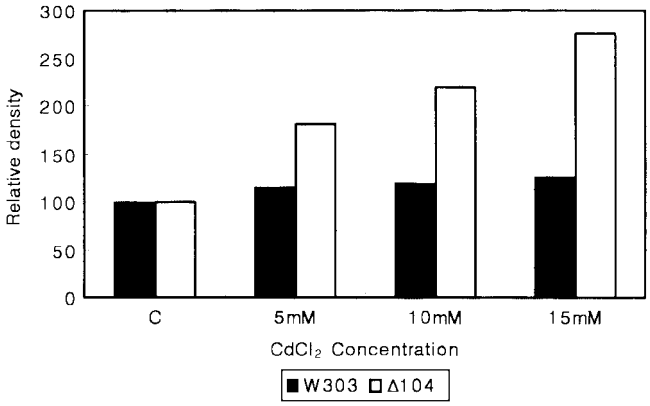

Figure 2. Estimation of ROS production resulting from cadmium exposure. After treated with cadmium, the level of ROS generation was measured with oxidation of H_2DCF. Fluorescence was measured with excitation and emission wavelengths of 504 and 524 nm, respectively.

(H_2DCF).[16] This nonfluorescent probe enter the cell and is hydrolyzed to fluorescein and acetate by nonspecific esterase, acidic pH or oxidants, such as ROS. As expected, wild type cells did contain little amount of ROS, thereby showing an insignificant sensitivity against cadmium toxicity (Figure 2). On the other hand, with hsp104 gene deleted, Δ104 produced significantly increasing amount of ROS in a dose dependent manner. The difference in ROS production upon cadmium treatment between with or without Hsp104 protein indirectly suggests that cadmium stress mechanism may be regulated by oxidative stress pathway.

Importance of Two ATP Binding Sites in Protection of Cadmium Stress. It has been known that both of two ATP binding sites in Hsp104 protein are required for stress tolerance. Schimer et al. has reported that mutation in the first ATP binding site (G217V or K218T) severly reduces ATP hydrolysis but has the little effect on oligomerization. Similar mutation in the second ATP binding site (G619V or K620T) has insignificant effects on ATPase activity but impair oligomerization.[17] Even though the two ATP binding sites seem to play different roles as compared above, any mutant with either single site altered (K218T or K620T) or both sites altered exhibits the same degree of thermosensitivity as well as oxidative damage. In order to examine functional roles of two ATP binding sites in regulation of cadmium stress, we have studied the previous recombined yeast strains in which derivatives of Hsp104 were expressed (manuscript in preparation). Recombinant strains of SP1 and IR2.5 with the plasmid pYS104 (wild type Hsp104), pRS316 (negative control), pKT620 (2nd ATP-binding site mutated), and pKT218/620 (both ATP-binding sites mutated) were constructed and investigated for survival rates (Figure 3) and ROS productions (Figure 4 and 5). The SP1-derived strains showed that only when wild type Hsp104 was overexpressed by GPD promoter, cells obtained resistance against cadmium toxicity even at 2.0mM $CdCl_2$ (Figure 3A). Neither pKT620 nor pKT218/620 helped cells survive against cadmium exposure. Similarly, *ira2* deleted hyperactivated *ras* mutant became also more resistant in the presence of two ATP binding sites as in pYS104 than in the absence of ATP binding sites as in either pKT620 or pKT218/620 (Figure 3B). It is very interesting, however, that the degrees of protection in wild type derived strains were as same as that in *ras* mutant strains even if wild type cells exhibited higher sensitivity to oxidative stress than *ras* mutants (Figure 4A and Figure 5A). Based on these results we conclude that the presence of two ATP binding sites, regardless of Ras-cAMP pahway, are crucial in providing cadmium protection in yeasts.

Importance of Two ATP Binding Sites in Regulating ROS Production. In order to investigate cross-relationship between ATP binding sites, cadmium stress and oxidative stress, we have performed fluorescence tests using these constructs (Figure 4B and Figure 5B). In both wild type and mutant cases, cells harboring pYS104 generated ROS about 120% of control upon cadmium treatment whereas cells containing pRS316, pKT620 or pKT218/620 produced significant amounts of ROS up to 200% of control. These data imply that functional Hsp104 protein with both ATP binding sites present provides protection against cadmium stress via rescuing cells from oxidative stress. It may help yeast cells carry out metabolism normally so that they can survive even under stress conditions. As a result, either generation of ROS as byproducts from respiration pathway can be prevented or detoxification of ROS has been efficiently performed within cells.

Experimental Section

Yeast Strains. Studies were performed with wild type W303 (Mata *can1 his3 leu2 trp1 ura3 ade2*), mutant Δ104 (Mata *can1 his3 leu2 trp1 ura3 ade2 hsp104::LEU2*),[12] SP1 (*MATa his3 leu2 ura3 trp1 ade8*) and IR2.5 (*MATa his3 leu2 ura3 trp1 ade8 ira2::ADE8*).[18] YPD (or YPA) medium containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) dextrose (or acetate) was used for cell growth. DO-Ura contains 0.67% Bacto-yeast nitrogen base without amino acid (YNB) (Difco), 2% glucose supplemented with histidine, leucine, tryptophan and adenine at the final concentration of 0.2%. All the chemicals used for metal stress and spectroscopic analysis were purchased from either Sigma or Aldrich except for 2',7'-dichlorofluorosin diacetate from molecular probe.

Yeast Transformation. Both SP1 and IR2.5 grown in YPD were transformed with pYS104, pRS316, pKT620 or pKT218/620 by lithium acetate methods. Phenotypes of recombined cells were confirmed by thermotolerance test and western blotting (manuscript in preparation).

Metal Stress Test. Yeast cells were cultured overnight and diluted either in fresh YPA media. or DO-ura(gal) media. (O.D = 0.25 at 600 nm). Resuspended cells were divided into 4 equal part in vials and were exposed to each concentration of $CdCl_2$ for incubated time with shaking. 200 μL of each sample was transferred into a 96 well plate and stamped on a YPD plate after serial dilutions. Each YPD plate was

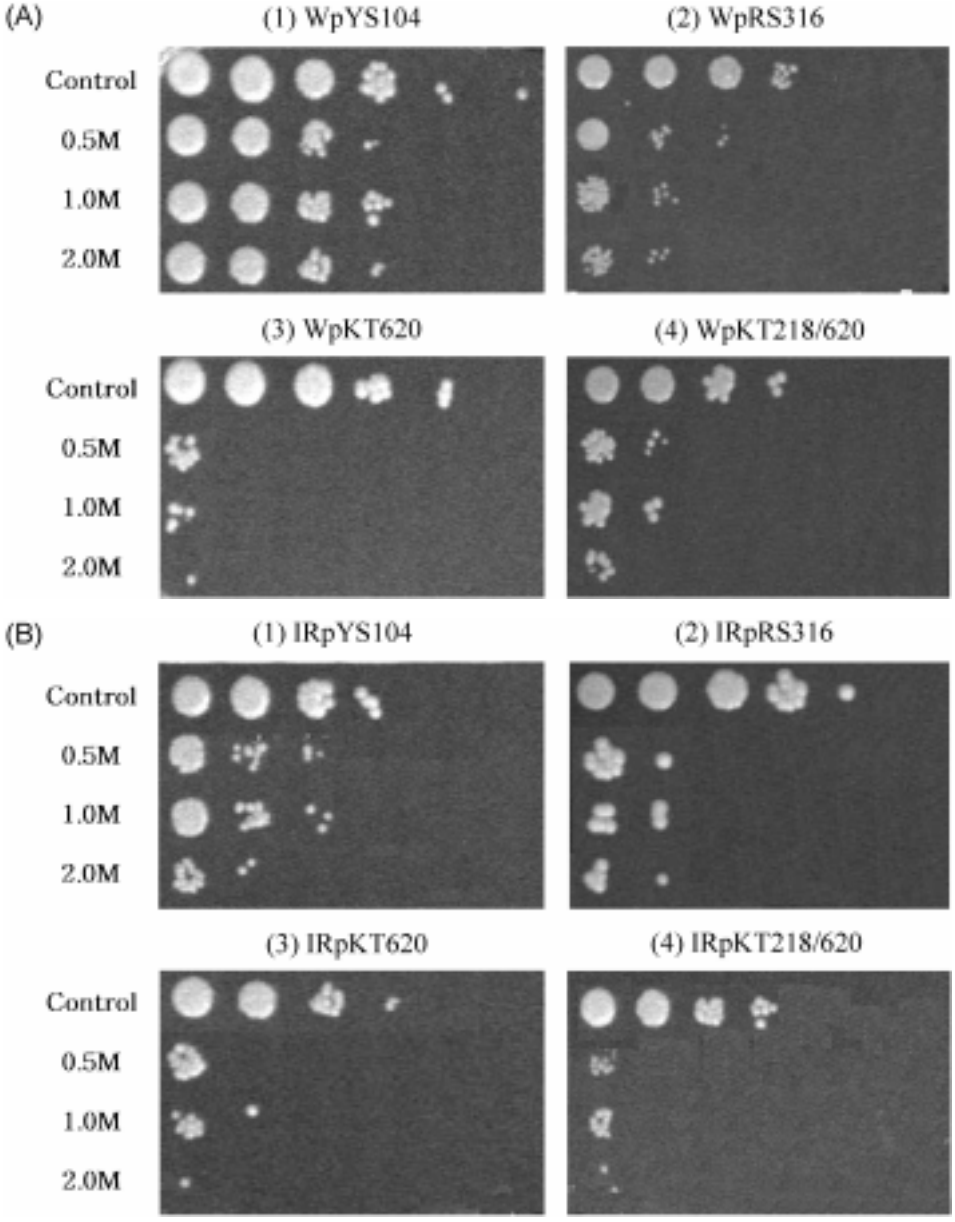

Figure 3. Sensitivity of (A) wild type-derived strains and (B) hyperactivated *ras* mutant-derived strains upon cadmium exposure at 0 mM, 0.5 mM, 1.0 mM and 2.0 mM, respectively. Plasmids inserted into wild type SP1 were (1) pYS104 expressing the wild type copy of Hsp104 under the GPD promoter, (2) pRS316 as a negative control vector, (3) pKT620 containing the mutated Hsp104 in the second ATP binding site ($Lys^{620} \rightarrow Thr^{620}$) and (4) pKT218/620 possessing $Lys^{218} \rightarrow Thr^{218}$ and $Lys^{620} \rightarrow Thr^{620}$ mutation at both ATP bindng sites.

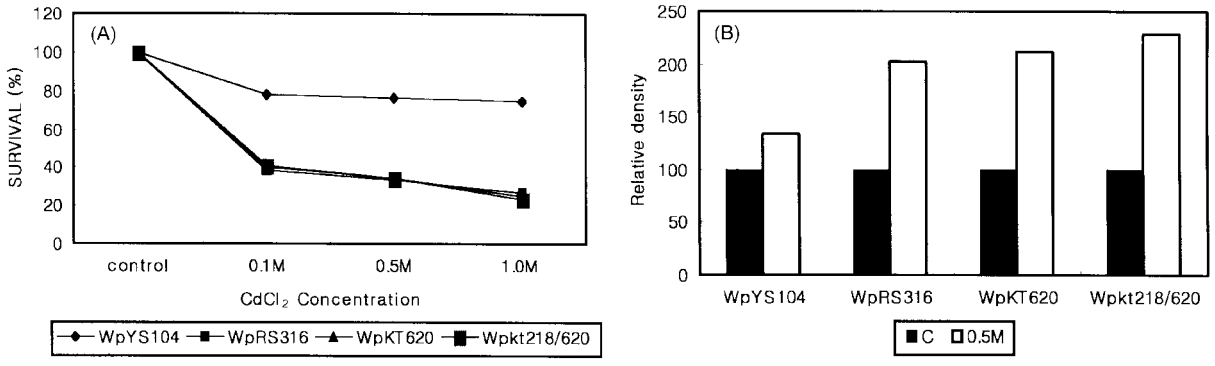

Figure 4. (A) Viability of cadmium treated wild type-derived constructs, WpYS104, WpRS316, WpKT620 and WpKT218/620. (B) Relative production of ROS after 0.5M $CdCl_2$ treatment measured by H_2DCF fluorescence tests.

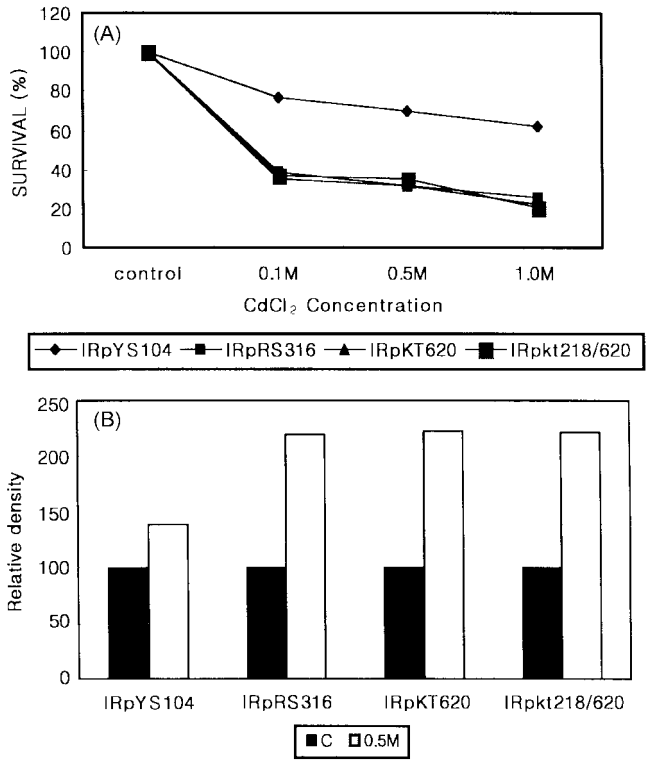

Figure 5. (A) Survival of cadmium treated hyperactivatd *ras* mutant-derived cells, IRpYS104, IRpRS316, IRpKT620 and IRpKT218/620. (B) Relative production of ROS in each strain after 0.5M $CdCl_2$ treatment measured by H_2DCF fluorescence tests.

incubated at 30 °C for 2 days and the colony-forming units were photographed by an image analyzer. For colony counting assays, 100 μL samples of cells after appropriate dilution was taken and spread on the surface of YPD plates so that the control plate contains 100-200 colonies. After incubation at 25 °C until visible growth has occurred, the colonies were counted. The number of viable cells in each plate was expressed as the percentage of control.

Measurement of ROS Production by Fluorescence. 20 μL yeast cells were diluted in 980 μL potassium buffer and 5 mM stock solution of 2',7'-dichlorofluorosin diacetate (H_2DCF) dissolved in distilled water (160 μL) was added to each culture.[19] Cell were incubated for 30 min with shaking and placed on ice, washed twice in ice-cold distilled water, and resuspended in 300 μL of water. After vortexed with 300 μL of glass beads (Sigma), supernatant was obtained after micro-centrifuged for 10 min. Resulting crude extract was suspended in distilled water and quantified for protein concentration using Bradford method.[20] Fluorescence was recorded with excitation wavelength of 504 nm and emission wavelength of 524 nm.

Acknowledgment. We thank Dr. R. Ballester (U. of California at Santa Barbara) for providing SP1 and IR2.5 mutant strains and Dr. S. B. Hwang for valuable discussions throughout this study. The authors also wish to acknowledge the financial support of the Korea Research Foundation made in the program year of 1998 (BSRI-98-3418).

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