Increased cellular resistance to oxidative stress by expression of cyanobacterium catalase-peroxidase in animal cells

Takahiro Ishikawa[a], Yuriko Ohta[a], Toru Takeda[b], Shigeru Shigeoka[b], Morimitsu Nishikimi[a,*]

[a] *Department of Biochemistry, Wakayama Medical College, Wakayama 640-8155, Japan*
[b] *Department of Food and Nutrition, Faculty of Agriculture, Kinki University, Nara 631-8505, Japan*

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Abstract To exploit prokaryotic antioxidant enzymes for protection of animal cells from oxidative damage, we expressed catalase-peroxidase of cyanobacterium *Synechococcus* PCC 7942 in 104C1 cells. The gene for this enzyme was inserted into the mammalian expression vector pRc/CMV. The stable transfectants obtained had higher specific activities of catalase and as a result became more resistant to H_2O_2 or paraquat than the parental cells. Subcellular fractionation and immunoblot analysis revealed that the expressed catalase-peroxidase was confined to the cytosol; this localization may be the basis for the effective protection of the transfectants from the oxidative cell damage.

Key words: Catalase-peroxidase; Oxidative stress; Expression; *Synechococcus* PCC 7942; 104C1 cell

1. Introduction

Many reactions occurring in cellular oxygen metabolism unavoidably generate reactive oxygen species, such as $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$, which are associated with various pathogenic processes, e.g. carcinogenesis, inflammation, radiation injury, and aging [1]. The most highly reactive among these species is $^{\bullet}OH$, which would be derived from H_2O_2 through the reaction with Fe^{2+} or Cu^{+}. Therefore, decomposition of H_2O_2 is pivotal in the protection of the cell from oxidative damage, and in fact, aerobic organisms possess catalase and peroxidase for detoxication of H_2O_2 [1]. Prevention of oxidative cell damage by an antioxidant enzyme has been tried by direct administration of the enzyme itself both in vitro and in vivo. A more challenging approach is to introduce a gene for the enzyme into cells by gene transfer technique. Catalase is a choice among antioxidant enzymes because it needs no other substrate than H_2O_2 for its enzymic reaction. However, its H_2O_2-scavenging ability is limited in terms of a poor affinity for H_2O_2 ($\sim$1 M) and its discrete localization in peroxisomes.

Recently, we purified catalase-peroxidase (CPX) of the cyanobacterium *Synechococcus* PCC 7942 (*S.*7942) and also isolated its gene [2]. This enzyme is composed of two identical subunits with a molecular mass of 79 kDa and characterized by a low K_m value for H_2O_2 (4.6 mM). In this study, we have employed this gene to confer resistance to oxidative stress on animal cells by gene transfer technique. Because this enzyme has no transit signal sequence to any organelles, it could be localized in the cytosolic compartment when expressed in animal cells. Guinea pig 104C1 cells harboring an expression plasmid for the CPX gene have been shown to produce CPX with the expected localization and to increase the resistance to treatment not only with H_2O_2 but also with paraquat.

2. Materials and methods

2.1. Materials

The guinea pig cell line 104C1, established by Evans et al. [3], was obtained from the Japanese Cancer Research Resources Bank. *Pfu* DNA polymerase was purchased from Clontech, Palo Alto, CA; pRc/CMV vector from Invitrogen, San Diego, CA; and M13 reverse primer from Toyobo, Osaka, Japan. RPMI 1640, cell culture supplements, and fetal calf serum were from Gibco BRL, Grand Island, NY. All other chemicals were of the highest grade of purity commercially available.

2.2. Construction of expression vector for S.7942 CPX gene

A previously obtained clone encoding the *S.*7942 CPX gene (designated pSCP2) [2] was used as a template for PCR amplification. A sense primer (5′-AGACTGCCACCATGACAGCAACCCAGG-3′) corresponding to nucleotides 3–29 was synthesized such that the Kozak translational starting motif [4] was introduced around the initiation codon, and M13 reverse primer was used as an antisense primer. The PCR amplification was performed in 50 μl reaction mixtures containing 1.0 μM each of the primers, 1.25 units of *Pfu* DNA polymerase, 200 μM dNTPs, 20 mM Tris-HCl (pH 8.2), 2 mM $MgCl_2$, 10 mM KCl, 6 mM ammonium sulfate, 0.1% Triton X-100, and 10 μg/ml bovine serum albumin. The PCR cycle consisted of 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The PCR product was cleaved at the *Not*I site located between the 3′ end of the gene and the site of M13 reverse primer, and ligated to the *Eco*RV/*Not*I site of pBluescript SK^+. The insert DNA was excised from the resulting plasmid with *Hin*dIII and *Not*I, and ligated to the respective sites of pRc/CMV, so that the CPX gene could be expressed under the control of the CMV promoter (Fig. 1). The final construct (designated pRc/CPX) was used to transform *Escherichia coli* JM 109, and the plasmid was purified by CsCl density gradient centrifugation.

2.3. Transfection of 104C1 cells

Cells of the guinea pig cell line 104C1 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37°C under atmosphere of 5% CO_2 and 95% air. Exponentially growing cells were transfected with pRc/CPX by the calcium phosphate method [5]. The transfected cells were subsequently selected in the RPMI 1640 medium containing 400 μg/ml G418 by exchanging the medium every 3 days for 2 weeks, and individual G418-resistant colonies were isolated using cloning cylinders. Thereafter, the cloned cells were cultured in the RPMI 1640 medium containing 200 μg/ml G418.

2.4. Enzyme assays

Cells were washed with Dulbecco's modified phosphate-buffered saline without Mg and Ca (PBS), suspended in 50 mM sodium phosphate buffer (pH 7.0), and sonicated in an ice-water bath by three 15 s bursts with 30 s cooling intervals; and the resulting suspension was

*Corresponding author. Fax: (81) (734) 26-8367.
E-mail: nishikim@seishu.wakayama-med.ac.jp

Abbreviations: CPX, catalase-peroxidase; *S.*7942, *Synechococcus* PCC 7942; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide

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centrifuged at 12 000 × *g* at 4°C for 10 min. The supernatant was used for enzyme assays.

Catalase activity was determined spectrophotometrically by measuring the decrease in absorbance at 240 nm in a 1.0 ml solution containing 50 mM sodium phosphate buffer (pH 7.0), 10.5 mM H_2O_2, and enzyme at 27°C [2]. One unit of enzyme was defined as the quantity that catalyzes the decomposition of 1 μmol of H_2O_2 in 1 min. Superoxide dismutase [6], glutathione peroxidase [7], and glutathione *S*-transferase [8] were assayed by the methods described in the cited references. The protein concentration was determined with Coomassie Protein Assay Reagent (Pierce, Rockford, IL).

2.5. Immunoblot analysis

Proteins were heated in a sample solvent containing 2-mercaptoethanol, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% gels) according to Laemmli [9], and blotted onto a cellulose nitrate membrane (Advantec, Tokyo, Japan) using a semidry electroblotting system (Taitec, Saitama, Japan). Visualization of *S*.7942 CPX was performed with its specific antibody as described previously [2].

2.6. Subcellular fractionation

The following procedures were carried out at 4°C. Cells were washed with PBS and homogenized in 10 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA, using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 10 000 × *g* for 3 min, and the resulting supernatant was recentrifuged at 100 000 × *g* for 30 min. Aliquots of subcellular organelle fractions containing an equal amount of catalase activity were subjected to immunoblot analysis as described above.

2.7. Cytotoxicity test with H_2O_2 and paraquat

pRc/CPX-transfected cells and the parental 104C1 cells were seeded into 6 well microplates at 2–4 × 10^4 cells/well in 2 ml of RPMI 1640 medium containing 10% fetal calf serum. After the cells had grown to semiconfluency, they were incubated with varying concentrations of H_2O_2 or paraquat in 2 ml of RPMI 1640 medium containing 1% fetal calf serum. After an appropriate period of incubation (3 h for H_2O_2 and 48 h for paraquat), the MTT assay was performed for determination of cell viability as described by Mosmann [10].

3. Results

3.1. Expression of S.7942 CPX in 104C1 cells

The *S*.7942 CPX gene was inserted into the mammalian expression vector pRc/CMV, after the Kozak consensus sequence [4] was introduced around the initiation codon (Fig. 1). The resulting expression plasmid (pRc/CPX) was used for transfection of 104C1 cells, and three G418-resistant stable transfectants (designated 104C1-CPX1, 104C1-CPX2, and 104C1-CPX3) were isolated. Transfectant 104C1-CPX2 had the highest catalase activity, followed by 104C1-CPX3 and 104C1-CPX1. The specific activity of catalase in these transfectants was about 2.0–6.6-fold greater than that in non-transfected 104C1 cells or in control cells transfected with pRc/CMV vector (Table 1). On the other hand, there was no significant difference in activities of other cellular antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, and glutathione *S*-transferase, among the pRc/CPX-transfected and non-transfected 104C1 cells (Table 1). Fig. 2 shows a result of immunoblot analysis for the CPX expressed in the three transfectants. All of their extracts gave positive bands at a position of the subunit molecular mass (79 kDa) of *S*.7942 CPX [2], whereas the non-transfected cells and the cells transfected with pRc/CMV vector showed no immunoreactive band. The content of CPX protein expressed in the three transfectants varied in parallel with the level of their catalase activity.

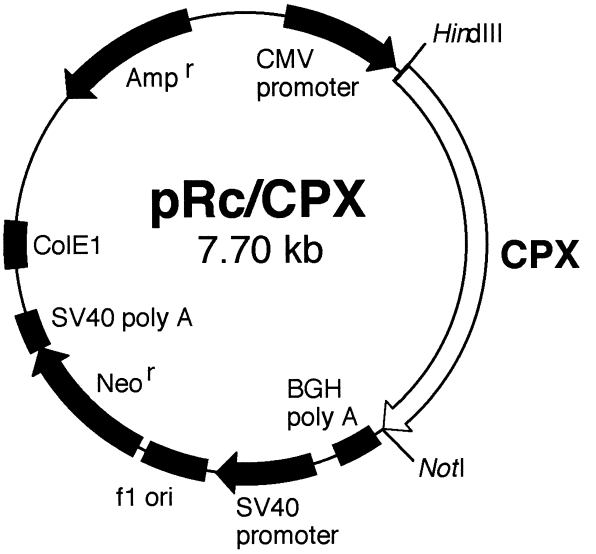

Fig. 1. Structure of the expression plasmid pRc/CPX for expression of *S*.7942 CPX. The PCR-amplified CPX gene was inserted into the multicloning site of pRc/CMV, so that it could be expressed under the control of the CMV promoter. The details of the construction are described in Section 2.

The localization of the CPX expressed in pRc/CPX-transfected 104C1 cells was examined by differential centrifugation of cell lysate from transfectant 104C1-CPX2. Catalase activity was detected in both post-100 000 × *g* supernatant and post-10 000 × *g* pellet fractions (data not shown). However, on immunoblot analysis, an immunoreactive band at a position of 79 kDa was observed only with the former fraction, and not with the latter fraction, when an equal amount of catalase activity (0.2 unit) from each fraction was tested (Fig. 3). This result indicates that the expressed CPX is localized only in the cytosolic compartment, and not in organelles such as peroxisomes.

Table 1
Activities of catalase and other antioxidant enzymes in 104C1 cells transfected with pRc/CPX

Cell line	Catalase[a]	Glutathione peroxidase[a]	Glutathione *S*-transferase[a]	Superoxide dismutase[b]
104C1	9.9 ± 0.9	0.05 ± 0.005	0.98 ± 0.03	95.5 ± 3.5
104C1-pRc/CMV	8.4 ± 0.6	0.06 ± 0.003	0.73 ± 0.05	110.1 ± 8.0
104C1-CPX1	16.7 ± 0.7	0.05 ± 0.004	0.85 ± 0.04	109.5 ± 6.1
104C1-CPX2	55.3 ± 2.4	0.06 ± 0.005	0.92 ± 0.05	106.7 ± 5.2
104C1-CPX3	39.6 ± 1.1	0.07 ± 0.004	0.90 ± 0.05	107.4 ± 7.6

[a]μmol/min/mg protein.
[b]units/mg protein.
Values are means ± S.D. of three independent experiments.

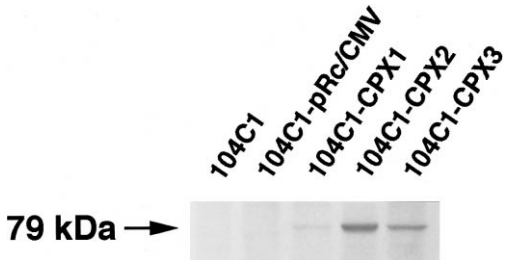

Fig. 2. Immunoblot analysis for the CPX expressed in transfectants with pRc/CPX. Cell extracts (50 μg protein) from three cloned transfectants and nontransfected 104C1 cells were subjected to SDS-PAGE in a 10% gel and analyzed by immunoblotting using anti-*S.*7942 CPX antibody.

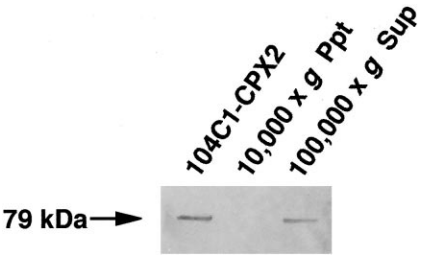

Fig. 3. Subcellular localization of the CPX expressed in transfectant 104C1-CPX2. Subcellular fractionation of cell lysate from transfectant 104C1-CPX2 was performed as described in Section 2. Aliquots of subcellular organelle fractions containing an equal amount of catalase activity (0.2 unit) were subjected to SDS-PAGE in a 10% gel and analyzed by immunoblotting using anti-*S.*7942 CPX antibody.

3.2. Resistance of the transfectants to H_2O_2 or paraquat

We used the isolated transfectants to examine whether the expressed CPX protects them from oxidative damage caused by H_2O_2 or paraquat. Before this experiment, we ascertained that there was no significant difference in cell growth rate among all transfectants and non-transfected 104C1 cells (data not shown). The cell viability after incubation with varying concentrations of H_2O_2 for 3 h was assessed by the MTT assay. The LD_{50} values were 280 μM for the non-transfected 104C1 cells, 370 μM for 104C1-CPX1, 820 μM for 104C1-CPX2, and 650 μM for 104C1-CPX3 (Fig. 4A), the cellular resistance to H_2O_2 being increased with an increase in the level of the expressed CPX. Next, transfectant 104C1-CPX2 with the highest catalase activity was tested for protection from paraquat toxicity. The LD_{50} of paraquat (48 h incubation) for the transfectant (640 μM) was much higher than that for the non-transfected 104C1 cells (180 μM) (Fig. 4B). It has been known that paraquat produces $O_2^{\bullet-}$ via a cyclic redox reaction with molecular oxygen and at the same time produces H_2O_2 through disproportionation of the $O_2^{\bullet-}$. The obtained results indicate that the CPX expressed in the cytosolic compartment suppresses cell damage caused by H_2O_2 that is generated inside the cell as well as by H_2O_2 that attacks the cell from the outside.

4. Discussion

The aim of our study is to increase cellular antioxidative capacity by genetic engineering of an antioxidant enzyme. We chose the CPX gene of *S.*7942, whose gene product possesses a higher affinity for H_2O_2 than mammalian catalase, and constructed a plasmid that renders animal cells able to express the CPX under the control of the CMV promoter. When guinea pig 104C1 cells were transfected with the plasmid, all three stable transfectants isolated had increased levels (2.0–6.6-fold) of catalase activity (Table 1). This is the first case to employ a gene of prokaryotic origin for functional expression of an antioxidant enzyme in animal cells.

A few recombinant systems for expression of mammalian catalase cDNAs have been reported regarding the effect of overexpressed catalase on cytotoxicity caused by oxidative stress. Two mouse epidermal cell transfectants with a human catalase cDNA had 2.6- and 4.2-fold increases in catalase activity and exhibited a resistance to extracellular burst of $O_2^{\bullet-}$ and H_2O_2 by xanthine oxidase reaction [11]. In contrast, L-cells that expressed human catalase about 100-fold were more sensitive than control cells to a variety of auto-oxidizable substances including paraquat [12]. Transgenic mice that overexpressed rat catalase in the heart were examined as to whether they become resistant to doxorubicin-induced cardiotoxicity [13]. Transgenic mice expressing 60- or 90-fold catalase activity compared with control mice showed a marked resistance, whereas those expressing 200-fold or more catalase activity did not. The transgenic mice expressing 60-fold catalase activity was also reported to be resistant to ischemia-reperfusion and hypoxia-reoxygenation injuries [14,15]. These

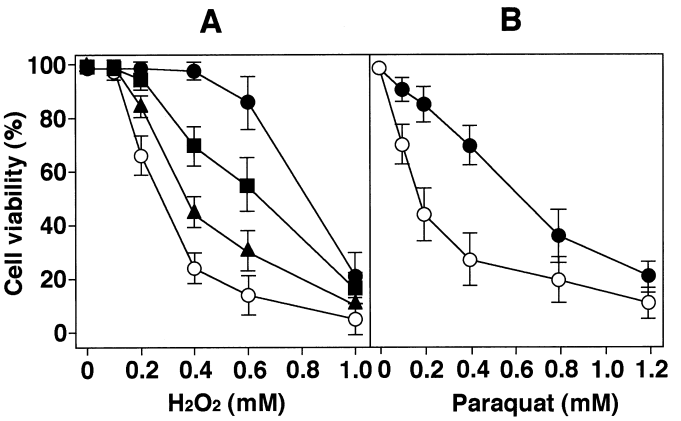

Fig. 4. Viability of transfectants with pRc/CPX after incubation with H_2O_2 (A) and paraquat (B). The transfectants (▲, 104C1-CPX1; ●, 104C1-CPX2; ■, 104C1-CPX3) and the parental 104C1 cells (○) were grown to semiconfluency in 6 well microplates and cultured in RPMI 1640 medium containing 1% fetal calf serum, in the presence of varying concentrations of H_2O_2 for 3 h or varying concentrations of paraquat for 48 h. The MTT assay was then performed to examine cell viability. Data are expressed as means ± S.D. of three independent experiments.

results, taken together, indicate that an excess of catalase present in the cell is not beneficial and that there is an optimal level for attaining enhancement of cellular antioxidative capacity. In our present study a transfectant with 6.6-fold catalase activity was markedly resistant to oxidative damage caused by H_2O_2 or paraquat (Fig. 4). It should be noted that even doubling of catalase activity significantly ($P < 0.05$) protected the cells from up to 0.6 mM H_2O_2 included in the culture medium. Because *S.*7942 CPX does not contain any transit signal sequences to organelles of animal cells [2], it was successfully expressed in the cytosolic compartment of transfected cells. This localization of the expressed CPX effects a broader distribution of H_2O_2-scavenging machinery in the cell. This may be one of the reasons for the effective protection by expression of the CPX. This efficient expression system could be a useful tool for testing involvement of H_2O_2 in pathophysiological processes associated with reactive oxygen species.

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