Short communication

Correlation between the Phosphohydrolase Activity of the *Escherichia coli* Orf135 (NudG) Protein and Mutation Suppression

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The *Escherichia coli* Orf135 (NudG) protein, a MutT-type enzyme, catalyzes the hydrolysis of 2-hydroxy-dATP and 8-hydroxy-dGTP, and its deficiency causes an increase in the mutation frequency. In this study, Orf135 proteins with substitutions at the Gly-36, Gly-37, Lys-38, Glu-43, Arg-51, Glu-52, Leu-53, Glu-55, and Glu-56 residues, which are conserved in three MutT-type proteins (Orf135, MutT, and MTH1), were each expressed in the *orf135*[−] strain, and the *rpoB* mutant frequency upon H₂O₂ treatment was examined. The *in vivo* mutation suppression abilities and the *in vitro* enzymatic activities obtained in a previous study were compared. The expression of the enzymatically active Orf135 mutants in the *orf135*[−] strain tended to reduce the *rpoB* mutant frequency induced by H₂O₂. This result suggests the importance of the phosphohydrolase activity in the suppression of mutations by the Orf135 protein.

Key words: Orf135, phosphohydrolase activity, mutation suppression, nucleotide pool sanitization

Introduction

Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen, are produced through normal cellular metabolism, and by various environmental mutagens and dietary factors (1–4). Base oxidation occurs in the cellular nucleotide pool as well as in DNA, and the oxidized deoxyribonucleotides induce mutagenic events (5). Specific hydrolysis of oxidized deoxyribonucleotides (nucleotide pool sanitization) is one of the mechanisms to prevent the mutagenesis caused by their misincorporation (6). The *Escherichia coli* MutT and mammalian MTH1 proteins catalyze the hydrolysis of 8-hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP) to the monophosphate derivative (7,8). Their importance has been shown by increases in the mutation frequency and tumor formation in organisms deficient in these proteins (9,10). Direct evidence that the MutT protein suppresses the mutagenesis caused by 8-OH-dGTP *in vivo* was shown by the significantly higher mutation-inducibility

of exogenous 8-OH-dGTP in the *E. coli mutT* strain than in the wild-type (wt) strain (11).

Previously, activities as nucleotide pool sanitization enzymes have been identified for two *E. coli* proteins, Orf135 (NudG) and Orf17 (NudB, NtpA) (12–14). The Orf135 protein catalyzes the hydrolysis of mutagenic deoxyribonucleotides, 2-hydroxy-2'-deoxyadenosine 5'-triphosphate (2-OH-dATP) and 8-OH-dGTP, *in vitro* (12,15,16). In addition, the lack of the Orf135 protein and its overexpression in *E. coli* cells result in increased and decreased mutation frequencies, respectively (17). The Orf135, MutT, and MTH1 proteins possess a homologous region, the “phosphohydrolase module” or “MutT signature” (18,19). The functional significance of the amino acid residues in this conserved region of the MutT and MTH1 proteins was analyzed (20–22). We previously measured the enzymatic activities of Orf135 mutants with various substitutions in the conserved amino acid sequence (23), and found that some of the mutant proteins had completely lost their activity, while others retained the activity to various degrees.

To know the correlation between the phosphohydrolase activity of the Orf135 protein and mutation suppression, we examined whether the expression of the mutant proteins suppressed the mutator phenotype observed for the *orf135*[−] strain. The results obtained in this study suggest the importance of this activity in the suppression of mutations *in vivo* by the Orf135 protein.

Materials and Methods

Materials: The *E. coli* strain JD22899, an *orf135*[−] strain (*lacI*^q, *lacZ*ΔM15-*gal*[−], *F*[−], *orf135*: mini Tn10 (*kan*)) (Miki *et al.*, unpublished results), was kindly provided by Drs. Yoshihiro Yamamoto and Takeyoshi

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Miki. Plasmid DNAs containing a mutant *orf135* gene as a fusion gene with GST (glutathione-S-transferase), constructed in the previous study (23), were transfected into the JD22899 strain.

Calculation of mutant frequency: A single colony of JD22899, harboring the gene for either the GST or GST-Orf135 fusion protein, was taken from an LB agar plate with kanamycin (10 μ g/mL), ampicillin (amp) (50 μ g/mL), and isopropyl- β -D-thiogalactopyranoside (IPTG) (2 μ M), and was inoculated into 7 mL of LB medium containing kanamycin, amp, and IPTG. The *E. coli* culture was incubated at 37°C until the turbidity at 570 nm reached 0.8, and then was diluted 10-fold with prewarmed LB medium containing amp and IPTG. When the turbidity at 570 nm reached 0.25, H₂O₂ was added to a final concentration of 2 mM, and the culture was incubated at 37°C for 30 min. The culture was centrifuged at 2,150 g for 15 min at room temperature. The pellet thus obtained was resuspended in the same volume of prewarmed LB medium containing amp and IPTG, and then was diluted 2-fold with prewarmed LB medium containing amp and IPTG. The *E. coli* culture was incubated at 37°C until the turbidity at 570 nm reached 0.85, and then was placed on ice for 10 min. A portion of the suspension was diluted with ice-cold LB medium and transferred onto an LB agar plate containing amp (a titer plate), which was incubated at 37°C for 12 h. Another portion of the suspension was transferred onto an LB agar plate containing rifampicin (100 μ g/mL) and amp (a selection plate), which was incubated at 37°C for 20 h. The mutant frequency was calculated according to the numbers of colonies on the titer and selection plates.

Results and Discussion

Mutant Orf135 proteins: Previously, we constructed a series of *orf135* genes encoding mutant proteins with substitutions of the amino acid residues that correspond to those essential for MTH1 to exert its 8-OH-dGTPase activity and are conserved in the Orf135, MutT, and MTH1 proteins (23). The Gly-36, Gly-37, Lys-38, Glu-43, Arg-51, Glu-52, Leu-53, Glu-55, and Glu-56 residues of Orf135 were each replaced with Ala, and some amino acids were substituted with their related ones. The wt Orf135 protein catalyzes the hydrolysis of 5-methyl-dCTP (5-Me-dCTP) and dCTP, in addition to 2-OH-dATP and 8-OH-dGTP (12,13,24). The mutant proteins with a substitution at the 36th, 37th, 52nd, and 56th amino acid residues completely lost their activity (23). On the other hand, the mutant proteins with a substitution at the 38th, 43rd, 51st, 53rd, and 55th residues could hydrolyze 5-Me-dCTP. In the current study, we examined the effects of the expression of the mutant Orf135 proteins on mutagenesis in the *orf135*⁻ strain.

Fig. 1. Suppression of H₂O₂-induced mutations by expression of the recombinant Orf135 protein. The *orf135*⁻ *E. coli* strains, harboring a plasmid for GST or GST-Orf135, were treated with 2 mM H₂O₂, as described in the Materials and Methods. Data are expressed as means $\pm$ standard deviation. Asterisks indicate a significant difference versus the GST plasmid, with $p < 0.05$ (*) or $p < 0.01$ (**).

Expression of the recombinant Orf135 protein suppresses mutations: A deficiency in the Orf135 protein causes an increase in the ROS (H₂O₂)-induced mutation frequency (17). We examined whether the expression of the mutant Orf135 proteins could suppress the mutator phenotype observed for the *orf135*⁻ strain. Plasmid DNAs containing the gene for either the GST or GST-Orf135 fusion protein were transfected into the *orf135*⁻ strain. The expression of these genes was induced by a treatment with 2 μ M of an inducer, IPTG. The protein production in the cells was confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (data not shown). The amounts of GST or the GST-Orf135 fusion proteins produced in cells were similar under the culture conditions that were used. These cells were treated with 2 mM H₂O₂, and their *rpoB* mutant frequencies were measured by counting colonies on rifampicin-containing agar plates.

The expression of the wt Orf135 protein in the *orf135*⁻ strain reduced the H₂O₂-induced mutant fre-

Fig. 2. Correlation between relative phosphohydrolase activity and mutant frequency. The relative enzymatic activity was defined as described in the text. (A) The wt and mutant GST-Orf135 proteins and the GST protein. (B) The wt, K38R, E43A, L53A, and E55Q mutant Orf135 proteins without the GST tag.

quency, as compared with that of the GST protein (Fig. 1), as reported previously (17). In addition, some of the mutant Orf135 proteins decreased the *rpoB* mutant frequency. The degrees of the reduction in the H_2O_2 -induced mutant frequency varied between the mutants tested (Fig. 1). The expression of the K38A, R51A, L53A, E55A, E55Q mutant proteins in the *orf135*⁻ strain significantly reduced the H_2O_2 -induced mutant frequency. These mutant proteins were enzymatically active *in vitro* (23).

Correlation of phosphohydrolase activity and mutation suppression: Next, we examined the relationship between the *in vitro* phosphohydrolase activities of the mutant Orf135 proteins fused to GST (23) and the H_2O_2 -induced *rpoB* mutant frequencies when they were expressed in *orf135*⁻ bacteria (Fig. 1). The 5-Me-dCTPase and dCTPase activities were measured with the GST-Orf135 proteins, purified on a small scale (Fig. 3 in reference 23). These activities of each mutant protein were normalized to those of the wt protein (wt = 1.0), and the averages of the normalized 5-Me-dCTPase and dCTPase activities were defined as the relative enzymatic activities. As shown in Fig. 2A, there is a correlation between the relative enzymatic activities and the H_2O_2 -induced mutant frequencies. The Pearson's correlation coefficient was calculated to be -0.47 , and this value is statistically significant ($p < 0.05$).

Previously, the K38R, E43A, L53A, and E55Q mutant proteins (without the GST tag) were examined in detail for their 5-Me-dCTPase, dCTPase, 2-OH-dATPase, and 8-OH-dGTPase activities (Fig. 4 in reference 23). These activities of each mutant protein were normalized to those of the wt protein (wt = 1.0), and the averages of the normalized hydrolysis activities were

defined as the relative enzymatic activities. Again, we analyzed the relationship between the relative enzymatic activities of the wt and the four mutant proteins, and the mutant frequencies when they were expressed in *orf135*⁻ bacteria (Fig. 1). As shown in Fig. 2B, a good correlation exists between the enzymatic activities and the H_2O_2 -induced mutant frequencies. The Pearson's correlation coefficient was calculated to be -0.96 , and it is statistically significant ($p < 0.05$). Significant ($p < 0.05$) correlations were also observed when each of the 5-Me-dCTPase, dCTPase, 2-OH-dATPase, and 8-OH-dGTPase activities was used as the phosphohydrolase activity (the correlation coefficients were -0.94 , -0.96 , -0.95 , and -0.89 , respectively; data not shown). Thus, the results of these experiments suggest that the Orf135 protein could suppress the ROS-induced mutagenesis derived from oxidized deoxyribonucleotide(s), through their elimination by its phosphohydrolase activity.

The weaker correlation between the H_2O_2 -induced mutant frequencies and the phosphohydrolase activities of the mutant GST-Orf135 proteins may be explained by the fact that the *in vitro* enzyme assay was not optimized. Alternatively, the possible presence of unknown substrate(s) in *E. coli* cells treated with H_2O_2 may explain the weaker correlation between the mutant frequencies and the phosphohydrolase activities of the mutant GST-Orf135 proteins. Since 8-hydroxy-dATP and formamidopyrimidine-dGTP were not hydrolyzed by Orf135 and were nonmutagenic in *E. coli* (25, Kamiya *et al.*, unpublished results), they are not candidates for the unknown substrate(s).

In this study, a significant relationship between the H_2O_2 -induced mutant frequencies and the phospho-

hydrolase activities of the mutant Orf135 proteins *in vitro* was demonstrated. Our results suggest that the Orf135 protein suppressed the ROS-induced mutagenesis derived from oxidized deoxyribonucleotides in *E. coli* cells, through their elimination by its phosphohydrolase activity.

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