In vivo glycosylation suppresses the aggregation of amyloidogenic hen egg white lysozymes expressed in yeast

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Abstract The mutant hen egg white lysozymes Ile55Thr and Asp66His, corresponding to human amyloidogenic mutant lysozymes Ile56Thr and Asp67His, respectively, were secreted in *Saccharomyces cerevisiae*. The amyloidogenic mutants (I55T and D66H) of hen egg white lysozymes were remarkably less soluble than that of the wild-type protein. To enhance the secretion of these mutants, we constructed the glycosylated amyloidogenic lysozymes (I55T/G49N and D66H/G49N) having the *N*-glycosylation signal sequence (Asn-X-Ser) by the substitution of glycine with asparagine at position 49. The secretion of these glycosylated mutant proteins is greatly increased in *S. cerevisiae*, compared with that of non-glycosylated type. Both the glycosylated mutants retained about 40% enzymatic activity when incubated at pH 7.4 for 1 h at the physiological temperature of 37°C whereas the non-glycosylated proteins eventually lost all activity under these conditions. These results suggest that the glycosylated chains could mask the β-strand of amyloidogenic lysozymes from the intermolecular cross-β-sheet association, thus improving the solubility of amyloidogenic lysozymes.

Key words: *N*-glycosylation; Amyloidosis; Hen egg white lysozyme; *Saccharomyces cerevisiae*

1. Introduction

The extracellular deposition of abnormal protein fibrils derived from soluble precursor proteins as amyloid fibrils is associated with serious diseases including systemic amyloidosis, Alzheimer's disease and transmissible spongiform encephalopathy [1–4]. Recently, Booth et al. reported that the two naturally occurring human lysozyme variants were both amyloidogenic, causing autosomal dominant hereditary amyloidosis [5]. The two natural mutations are heterozygous for the single base changes which encode non-conservative amino acid substitutions, Ile56Thr and Asp67His, respectively [5]. The Ile56 is a pivotal residue (corresponding to Ile55 in hen egg white lysozyme) for the structural integrity of the lysozyme fold in that it links the two domains. Its importance is emphasized by its high conservation in the lysozyme sequences [5–7]. Substitution of Asp67 (corresponding to Asp66 in hen egg white lysozyme) with histidine destroys the network of hydrogen bonds that stabilizes the β-domain, resulting in a large and concerted movement of the β-sheet and the long loop within the β-domain away from each other [5]. It seems likely that this fact is an exciting finding for understanding the mechanism of human amyloid formation. This also provides a possibility to suppress the formation of amyloid fibrils and to improve the solubility of amyloidogenic lysozymes. So far, there is little detail information about the mechanism of amyloid fibrillogenesis and the suppression of the formation of amyloidosis.

It was reported that a considerable amount of amyloidogenic mutants of human lysozyme could be obtained in the expression system using *Saccharomyces cerevisiae* [7]. On the other hand, the corresponding amyloidogenic mutants of hen egg white lysozyme could be obtained only in a very small amount, suggesting the greater facility of the amyloid formation than that of human lysozyme. Therefore, there are no reports on the amyloidogenic mutants of hen lysozyme.

We have reported that the glycosylated lysozymes can be secreted in the yeast carrying the lysozyme cDNA having *N*-linked glycosylation signal sequence (Asn-X-Ser) by site-directed mutagenesis. Interestingly, the secretion amounts are greatly increased compared with the wild-type lysozyme and keep the soluble form even on heating up to 95°C [8,9]. This suggests the possibility of the high secretion of unstable mutants which are poorly secreted in yeast expressing system. Therefore, the introduction of *N*-linked carbohydrate chains was attempted in order to increase the solubility of amyloidogenic lysozymes. Thus, we attempted to secrete glycosylated amyloidogenic lysozymes in *S. cerevisiae*. This attempt must shed light on the solution of disease caused by amyloidosis.

2. Materials and methods

2.1. Materials

Restriction endonuclease, T4 DNA ligase, and alkaline phosphatase were purchased from Takara Shuzo Co. Ltd (Kyoto, Japan). The DNA sequencing kit and DNA blunting kit were also purchased from Takara Shuzo Co. Ltd (Kyoto, Japan). Quick change™ site-directed mutagenesis kit was purchased from Stratagene Ltd (Cambridge). Synthetic oligonucleotides were purchased from Kurabo (Osaka, Japan). CM-Toyopearl resin was a production of Toso (Tokyo, Japan). Ethylene glycol chitin for lysozyme assay was from Sigma.

2.2. Bacterial strains and plasmids

Escherichia coli XL-1 blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac (F′ proAB, $lacI^qZ$ ΔM15, Tn10 $(tet^r)^c$)), which was used as a host cell in all cloning experiments, was supplied by Amersham Japan. *S. cerevisiae* diploid strain W303-1b (Mat α ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1 his3-11, 15), used as a yeast expression strain of mutant lysozymes, was provided by Dr. Parlati, McGill

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University. *S. cerevisiae* AH22 (Mat α, Leu, His4, Cir$^{\pm}$), used as another yeast expression strain of mutant lysozymes, were provided by Dr. I. Kumagai, Tohoku University. The recombinant plasmid pKK-1, which contains a full-length hen egg white lysozyme cDNA, was provided by Dr. I. Kumagai, Tohoku University [8]. pYG100, an *E. coli* yeast shuttle vector, was provided by Dr. K. Matsubara of Osaka University. The yeast episomal vectors pRS423 and pRS426 were provided by Dr. R. Akada, Department of Applied Chemistry and Chemical Engineering, Yamaguchi University.

2.3. Construction of S. cerevisiae W303-1b and AH22 expression plasmids of the mutant hen lysozymes

The cDNA of mutant lysozymes was inserted into the *Sal*I site in the pYG100 vector between the GPD promoter and terminator. The pYG100 carrying mutant lysozyme genes was treated with *Hin*dIII. Thus, the fragment (1.7 kb) was obtained from the promoter to terminator region containing the mutant hen lysozyme cDNA. By using blunt end approach, these fragments were inserted into the multiclonal site of pRS426, the expression plasmid of yeast *S. cerevisiae* W303-1b; and the multiclonal site of pRS423, the expression plasmid of yeast *S. cerevisiae* AH22.

2.4. Expression of the mutant hen lysozymes in S. cerevisiae W303-1b

The expression vector was introduced into *S. cerevisiae* W303-1b according to the lithium acetate procedure. Ura^{+} transformants were screened by subculturing in yeast minimum medium (JMM) supplemented with 20 μg/ml of histidine, 40 μg/ml of adenine, 60 μg/ml of leucine, 40 μg/ml of tryptophan at 30°C. The overexpressing subclones with the highest levels of lysozyme activity were screened and propagated from single colonies and were inoculated into 3 ml of JMM and incubated for 2 days at 30°C with shaking. This preculture was subcultured to 100 ml of the same medium in flask (500 ml) and incubated another 2 days at 30°C with shaking, and then 10 ml of second preculture was transferred to fresh 1 l of JMM in 3 l of flask and cultured at the same condition.

2.5. Expression of mutant hen lysozymes in S. cerevisiae AH22

The expression vector was also introduced into *S. cerevisiae* AH22 according to the lithium acetate procedure. His^{+} transformants were screened by subculturing in modified Burkholder's minimum medium (BMM) supplemented with 60 μg/ml of leucine at 30°C. After cultivation, the same method as that described for W303-1b was used in AH22 to express the mutant lysozymes.

2.6. Purification of the mutant hen lysozymes

The growth medium of the host cell was centrifuged at 7000 rpm for 10 min at 4°C. The supernatant was applied to a CM-Toyopearl 650 M column (1.5×5.0 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.5), and then adsorbed proteins were step-eluted using the same buffer containing 0.5 M sodium chloride [8]. Fractions containing the protein were collected, and then the samples which were desalted and concentrated by centricon centrifugal concentrators (Amicon, USA) were used in the experiments.

2.7. Endo H treatment of glycosylated amyloidogenic lysozymes

The method of Tarentino and Maley (1974) was slightly modified for the digestion of glycosylated amyloid-type lysozyme with endo-α-*N*-acetyl glucosaminidase H (*Endo H*). The glycosylated lysozymes were boiled in 50 mM sodium citrate buffer (pH 5.5) containing 1% SDS and 200 mg/ml of phenylmethylsulfonyl fluoride for 5 min. After cooling, samples were supplemented with an equal volume of either 0.02 unit of *Endo H* in 50 mM sodium citrate buffer (pH 5.5), and subsequently incubated at 37°C for 20 h. After incubation, the samples were analyzed by SDS–PAGE.

2.8. Chemical analysis

The total hexose content of the glycosylated lysozymes was estimated using the phenol-sulfuric acid reaction employing mannose standard. HPLC analysis serves as a method of identification of the hexose liberated from glycosylated lysozymes by hydrolysis with 2 N HCl at 100°C for 3 h in a sealed glass ampoule. The hydrolysates were dried, dissolved in water, and chromatographed on an Asahipak NH2P-50 column (Asahi Chemical, Tokyo) in 75% acetonitrile using the Hitachi HPLC system equipped with an RI detector. For the analysis of hexosamine, the hydrolysates with 3 N HCl at 100°C for 4 h were dried and then analyzed using an amino acid analyzer (Tosoh, HLC805).

2.9. Enzyme assay

Lysozyme activity was measured by glycolysis assay using ethylene glycol chitin as substrate. To 0.5 ml of lysozyme solution in 10 mM acetic acid sodium acetate buffer (pH 4.5) was added 1.0 ml of 0.05% solution of ethylene glycol chitin. The mixture was incubated at 40°C for 30 min. After the reaction, 2 ml of the color reagent (made by dissolving 0.5 g potassium ferricyanide in 1 l of 0.5 M sodium carbonate) was added, and the mixture was immediately boiled for 15 min to estimate the reducing power resulting from hydrolysis of ethylene glycol chitin.

2.10. Conformational stability of amyloidogenic hen lysozymes

The far-ultraviolet (200–260 nm) CD spectra were measured to determine the conformation change in mutant lysozymes. The protein concentration was adjusted to 0.05 mg/ml in 50 mM glycine–HCl buffer (pH 3.5). CD spectra were recorded by Jasco J-600 spectropolarimeter using 1.0 cm cuvette with maintaining temperature at 25°C. The value of CD spectra was expressed in terms of mean residue ellipticity (° cm^2 dmol^{-1}). The thermal denaturation of mutant lysozyme was measured by monitoring the changes in the ellipticity of CD spectrum at 222 nm. The protein solution was prepared in 50 mM glycine–HCl buffer (pH 3.5) at a concentration of 0.05 mg/ml. The thermal denaturation curves were drawn as function of the apparent fraction (f_{app}) of denatured proteins during heating. The f_{app} was calculated from the changes in the ellipticity at 222 nm.

3. Results and discussion

3.1. Secretion of amyloidogenic hen lysozymes

The amyloidogenic mutant lysozymes (I55T and D66H) were secreted into the culture medium of *S. cerevisiae* AH22 and W303-1b carrying the lysozyme expression vectors. The secreted proteins in the medium were collected and purified by the cation-exchange chromatography on CM-Toyopearl. Fig. 1 shows the secretion amounts of two amyloidogenic mutants and wild-type lysozyme in *S. cerevisiae* AH22 and W303-1b. As shown in the figure, the secretion of amyloidogenic lysozymes is extremely small. The amyloidogenic mutant lysozymes I55T and D66H may be remarkably less soluble than wild-type lysozyme in both yeast strains. In the case of *S. cerevisiae* W303-1b, the secretion of amyloidogenic lysozymes could not be observed. Although attempts to detect the lyso-

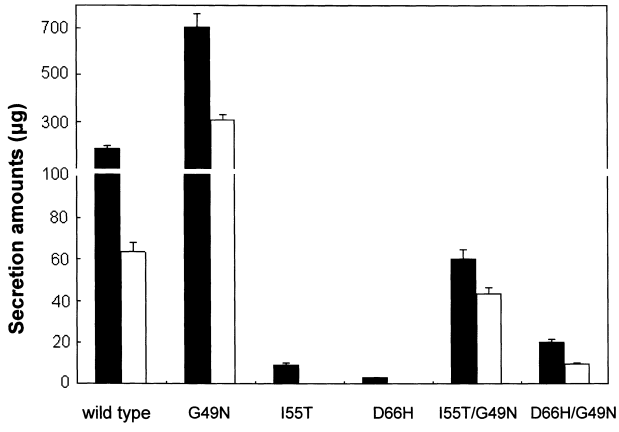

Fig. 1. Secretion amounts of wild-type, amyloidogenic mutant (I55T, D66H) and glycosylated mutant lysozymes (G49N, I55T/G49N and D66H/G49N). Lysozymes expressed in *S. cerevisiae* AH22 (black column) and *S. cerevisiae* W303-1b (white column). The vertical axis indicates the secretion amount per 1 l of growth medium. The vertical bars indicate the standard deviations ($n=3$) of secretion amounts.

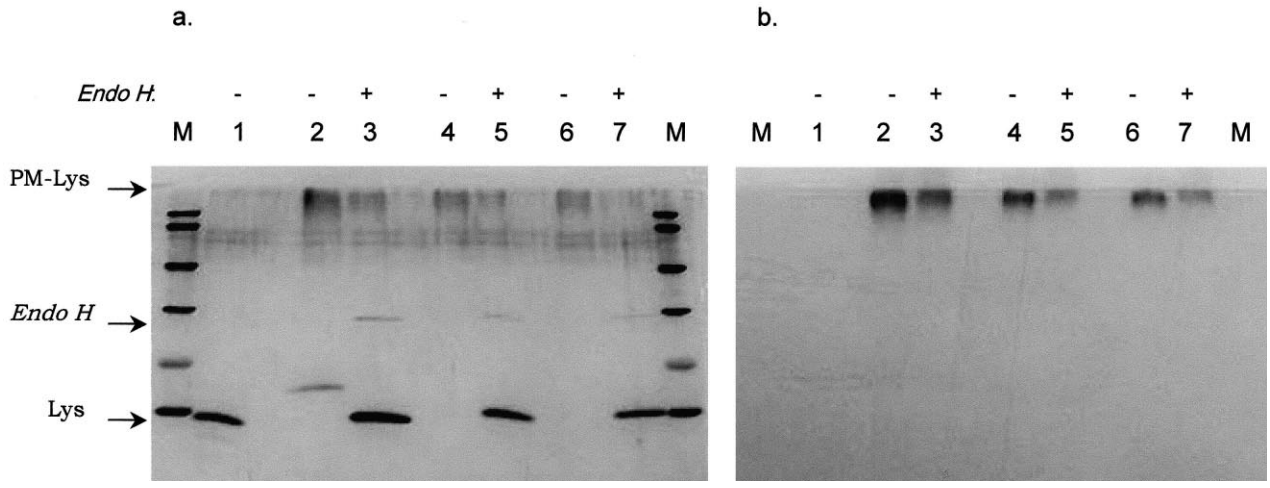

Fig. 2. SDS–PAGE patterns of glycosylated mutant lysozymes secreted in *S. cerevisiae* AH22. The eluates of unstable mutants (I55T/G49N, D66H/G49N) were concentrated to five times and those of stable mutants (G49N) were not concentrated before applying to electrophoresis. The gel sheets were stained for proteins and carbohydrates with silver stain kit Wako (a) and 0.5% periodic acid-Fuchsin solution (b), respectively. Lane 1, native lysozyme; lanes 2 and 3, G49N mutant lysozymes; lanes 4 and 5, I55T/G49N mutant lysozymes; lanes 6 and 7, D66H/G49N mutant lysozymes. The samples of lanes 3, 5, 7 were treated with *Endo H*. M, molecular marker (94 kDa, phosphorylase b; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, trypsin inhibitor; 14 kDa, lysozyme); PM-Lys, polymannosyl lysozymes; Lys, lysozymes.

zymes retained in the cell extract were made, the amounts were too small to detect, suggesting that most of the lysozymes were secreted outside the yeast cells. From these results, the secretion amounts of both amyloidogenic lysozymes were much lower than that of wild-type HEWL in the culture medium. It seems likely that amyloidogenic mutant lysozymes are aggregated after the secretion or are degraded by the quality control system of yeast.

3.2. Secretion of glycosylated amyloidogenic hen lysozymes

It has been proposed that the amyloidogenic lysozymes must have undergone major conformational change in vivo to form characteristic cross-β-amyloid fibrils through β-domain [5]. In order to suppress the formation of cross-β-amyloid fibrils, the introduction of carbohydrate chain was attempted in the exposed surface region at position 49 by site-directed mutagenesis (G49N). The glycosylated amyloidogenic mutant lysozymes were secreted into the culture medium of *S. cerevisiae* AH22 and W303-1b strain carrying the mutant lysozyme expression vectors. The secreted proteins in the medium were collected and purified by the cation-exchange chromatography on CM-Toyopearl. Fig. 1 shows secretion amounts of I55T/G49N and D66H/G49N mutants. The secretion amounts of glycosylated amyloidogenic lysozymes were much higher than that of the non-glycosylated amyloidogenic mutants. The secretion amounts of glycosylated amyloidogenic mutants are about six to seven times of non-glycosylated amyloidogenic proteins in *S. cerevisiae* AH22. Similarly, the increases in the secretion amounts of I55T/G49N and D66H/G49N were observed in the secretion system in another yeast strain W303-1b. The eluates obtained from *S. cerevisiae* AH22 were concentrated to five times and applied to SDS–PAGE and then the gel sheet was stained for protein and carbohydrate with silver stain kit Wako and 0.5% periodic acid-Fuchsin solution, respectively (Fig. 2). The molecular weight of glycosylated lysozymes was about 85 kDa with large carbohydrate chains. In another *S. cerevisiae* W303-1b strain, the similar result was also obtained (data not shown). From the carbohydrate analysis, about 310 moles of mannose appear to link to one molecule of lysozyme for the large glycosylated lysozyme. The carbohydrate content is almost the same as that of G49N mutant lysozyme [8].

3.3. Conformational stability of glycosylated amyloidogenic hen lysozymes (I55T/G49N, D66H/G49N)

The conformational stabilities of glycosylated amyloidogenic lysozymes were estimated by using CD analysis. Fig. 3 shows far-UV CD spectra to characterize the secondary structure of the glycosylated amyloidogenic lysozymes I55T/G49N and D66H/G49N. Compared with the wild-type lysozyme, the significant shift of the minimal value of CD spectra from 212 to 208 nm suggests the tendency of unfolding of the amyloidogenic mutants. The depths of 222 and 218 nm of CD reflect the content of α-helix and β-strand, respectively. The slight decreases in the absorbance at 222 nm, suggest the decrease in α-helix. The CD spectra of non-glycosylated amyloidogenic lysozymes I55T and D66H have the similar curve as the glycosylated ones (data not shown).

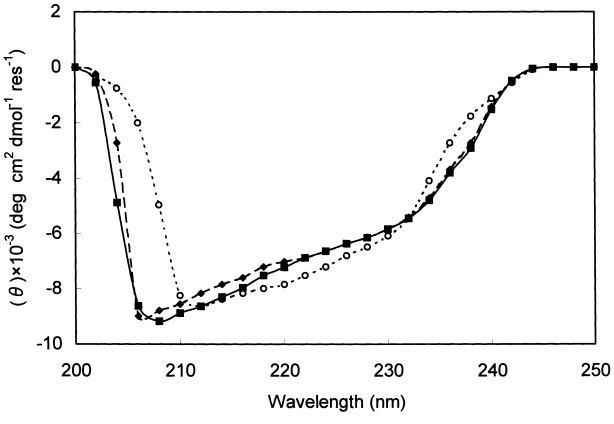

Fig. 3. Far-UV CD spectra for wild-type lysozymes (○), glycosylated amyloidogenic lysozymes I55T/G49N (■) and D66H/G49N (♦).

The thermal denaturation curves for these mutants were measured by monitoring the changes in the ellipticity of the spectrum at 222 nm during heating 35–80°C. Fig. 4 shows the typical denaturation curves for I55T, D66H, I55T/G49N and D66H/G49N. As shown in the figure, the unfolding transition midpoints for wild-type, I55T and D66H are 68.5, 55.7 and 50°C, respectively. The amyloidogenic mutants were less stable than wild-type lysozyme with unfolding transition midpoints 12°C or 18°C below that of wild-type protein. The reduction of unfolding transition midpoints of amyloidogenic mutants in the lysozyme is more profound than those in human lysozyme [5]. This is the reason why the amyloidogenic mutants in hen lysozyme are less soluble and unstable. The unfolding transition midpoints for I55T/G49N and D66H/G49N are 53.5°C and 46.9°C, respectively, suggesting further unstable conformation compared with non-glycosylated mutants.

The stability of glycosylated amyloidogenic hen lysozymes was further investigated by following the enzymatic activity incubated at pH 7.4 at 37°C for 1 h. The glycosylated amyloidogenic lysozymes were incubated at the physiological temperature of 37°C for 1 h, and then the solutions were centrifuged at 15 000 rpm for 10 min to remove the amyloid fibrils. The remaining lysozyme activities of supernatant were measured by glycolysis of glycol chitin. As shown in Table 1, both glycosylated amyloidogenic lysozymes retained 37 and 52% enzymatic activity for wild-type lysozyme, whereas the non-glycosylated proteins completely lost all activity under these conditions. This suggests that the glycosylation is effective to keep the soluble form of the amyloidogenic proteins.

It has been proposed for the possible mechanism of lysozyme amyloidosis that the soluble forms of amyloidogenic precursor proteins, through a transient population of intermediates with the structural characteristics of molten globules, convert to the amyloidosis through intermolecular β-sheet association [5]. The transient intermediates are thought to be of great importance for governing the formation of amyloid fibrils. The polymannosyl chain at position 49 resulting from the introduction of *N*-glycosylation sequence (Asn-X-Ser) must inhibit the cross-β-structure formation to the surface of lysozyme, thus preventing the formation of the amyloidogenic intermediates occurring in the intermolecular β-sheet association.

Hen egg white lysozyme has been well studied with respect to structural and functional properties. Therefore, there is a lot of information on wild-type and mutant lysozymes. A lot of unstable mutants have been reported to show the low melting temperatures and unstable conformation as well as amyloid-type mutants [10,11]. However, in our yeast expression system the secretion amounts of these mutants are much higher than of the amyloidogenic mutants (data not shown). A big difference is that amyloidogenic mutants are readily to aggregate, resulting low solubility. This strongly supports the hypothesis that the unstable proteins are not necessary to cause the amyloidosis, but the specific structural factor is essential for the formation.

As described in this paper, the polymannosyl chains could suppress the formation of amyloid fibrils and enhance the solubility of amyloidogenic lysozymes. Further studies are necessary to investigate whether the carbohydrate chain attachment is effective for the suppression of in vivo amyloidosis in higher eukaryotes. This attempt must shed light on the solution of disease caused by amyloidosis.

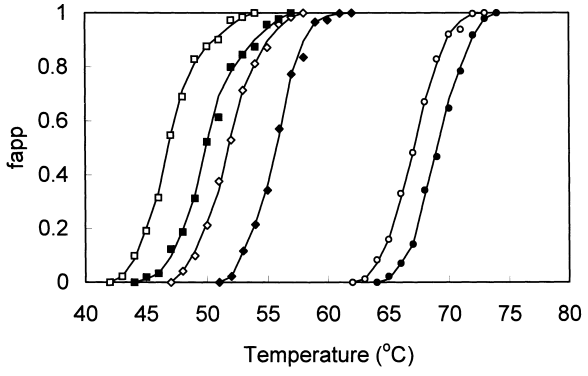

Fig. 4. Thermal denaturation curves of wild-type (●) and mutant G49N (○), I55T (◆), I55T/G49N (◇), D66H (■), D66H/G49N (□) lysozymes. The vertical axis indicates the ratio of denaturation.

Table 1
Remaining enzymatic activities of wild-type and mutant lysozymes after incubation for 1 h in 20 mM sodium phosphate buffer (pH 7.4) at 37°C

	Enzymatic activity (%)[a]
Wild-type	100
G49N	92
I55T	0
D66H	0
I55T/G49N	52
D66H/G49N	37

Each fraction purified on a CM-Toyopearl column chromatography was collected and the glycosylated activity was measured after incubation for 1 h in 20 mM sodium phosphate buffer (pH 7.4) at 37°C.
[a]Enzymatic activity was represented as the percentage of wild-type lysozymes. Each value is the mean deviation of three replications.

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