Transmembrane remote conformational suppression of the Gly-332 mutation of the Tn*10*-encoded metal-tetracycline/H^+ antiporter

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Abstract Gly-332 is a conformationally important residue of the Tn*10*-encoded metal-tetracycline/H^+ antiporter (TetA(B)), which was found by random mutagenesis and confirmed by site-directed mutagenesis. A bulky side chain at position 332 is deleterious to the transport function. A spontaneous second-site suppressor revertant was isolated from G332S mutant and identified as the Ala-354 → Asp mutant. Gly-332 and Ala-354 are located on opposite ends of transmembrane segment XI. As judged from [^{14}C]NEM binding to Cys mutants, the residue at position 354, which is originally exposed to water, was buried in the membrane by a G332S mutation through a remote conformational change of transmembrane segment XI. This effect is the same as that of a G62L mutation at position 30 through transmembrane segment II [Kimura, T., Sawai, T. and Yamaguchi, A. (1997) Biochemistry 36, 6941–6946]. Interestingly, the G332S mutation was also suppressed by the L30S mutation, and the G62L mutation was moderately suppressed by the A354D mutation. These results indicate the presence of a close conformational relationship between the flanking regions of the transmembrane segments II and XI.

Key words: Tetracycline; Drug resistance; Drug export; Site-directed mutagenesis

1. Introduction

The transposon Tn*10*-encoded tetracycline resistance protein (TetA(B)) is a metal-tetracycline/H^+ antiporter [2]. It actively effluxes tetracyclines using the H^+ concentration gradient across the membrane as an energy source [3,4]. It is a polytopic inner membrane protein whose 12 membrane-spanning structure was experimentally established by Kimura et al. [5] by means of site-specific chemical labeling of Cys-introduced mutants.

Functionally important residues of TetA(B) have been investigated by site-directed mutagenesis [6–11], and their distribution is mainly localized around transmembrane segments I, II and III, and segments VII, VIII and IX, and their flanking loop regions. In this study, new important residues were searched for in the whole TetA(B) protein by random mutagenesis. Random mutagenesis was performed with hydroxylamine [12]. When double-stranded DNA molecules are treated with hydroxylamine, a 4-amino group of cytosine is hydroxylated. The resulting hydroxylaminocytosine is matched to adenine when DNA is replicated. Therefore, a C-G base pair is randomly changed to a T-A base pair. On random mutagenesis, we found a mutation of Gly-332, which is located in cytoplasmic loop 10–11 in the putative topology (Fig. 1). The position of Gly-332 in the putative topology of TetA(B) resembles that of Gly-62, that is, both are located at the cytoplasmic end of a transmembrane segment, the second segment from the C-terminal and N-terminal ends, respectively. In our previous study, we showed that a bulky side chain at position 62 inhibits activity [13]. In addition, mutation of the residue at position 62 to one having a bulky side chain is suppressed by a second-site suppressor mutation of Leu-30, which is opposite to Gly-62 across the membrane [1]. In this study, we found that the mutation of Gly-332 was also suppressed by a mutation at the opposite side of the membrane. In addition, the mutations of Gly-62 and Gly-332 were mutually suppressed by the other's suppressor mutations, indicating a close conformational relationship between the regions flanking the second transmembrane helices from the N- and C-terminal ends.

2. Materials and methods

2.1. Materials

N-[Ethyl-1-^{14}C]maleimide (1.5 GBq/mmol) was purchased from DuPont-New England Nuclear, USA. The oligonucleotides used for site-directed mutagenesis were synthesized by Sawady Technologies (Tokyo, Japan). All other materials were of reagent grade and obtained from commercial sources.

2.2. Bacterial strains and plasmids

Escherichia coli CJ236 [14] were used for oligonucleotide-directed mutagenesis by the Kunkel method [14]. *E. coli* TG1 [15] and W3104 [16] were used for transformation after mutagenesis and expression of the *tet* gene, respectively. pCT1183 [17] and pLGT2 [10] are high copy number and low copy number plasmids, respectively, carrying 2.45 kb Tn*10-tetA(B)* and *tet*R gene fragments. pCT1183 and pLGT2 were used for mutagenesis and expression of the *tetA(B)* gene, respectively.

2.3. Random mutagenesis

A low copy number plasmid, pLGT2 (Km and TC), which encodes the *tet*R and *tetA(B)* genes, was treated with 0.4 M hydroxylamine in 50 mM sodium phosphate buffer (pH 6.0) at 70°C for 1 h. After transformation of *E. coli* TG1 cells with the resulting plasmids, colonies resistant to 50 μg/ml kanamycin and sensitive to 50 μg/ml tetracycline were picked up by replica plating. TetA(B) expression was judged on Western blotting of the sonicated membranes using anti-TetA(B)-C-terminal-peptide antiserum. In order to limit the mutation site, *Eco*RV–*Eco*RI (485 bp) and *Eco*RI–*Bam*HI (868 bp) fragments of these mutant plasmid DNAs, which encode the N-terminal half (Ile-39–Ser-199) and C-terminal half (Asn-200–Ala-401) of TetA(B), respectively, were isolated and recombined with the corresponding fragments of pLGT2. After the measurement of the drug resistance of these recombinant plasmids in *E. coli* TG1, the mutation sites were assigned by DNA sequencing.

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2.4. Site-directed mutagenesis

Site-directed mutagenesis was performed by the Kunkel method [14] using pCT1183 as a template, and the synthetic oligonucleotides containing mismatches generating a codon change and silent mismatches generating new restriction sites in order to check the mutations. Mutations were first detected as the appearance of new restriction sites and then verified by DNA sequencing using an ALFexpress DNA sequencer (Pharmacia Biotech). Then the mutant *tetA(B)* gene was transferred into pLGT2 through corresponding fragment exchange.

2.5. Determination of tetracycline resistance

The tetracycline resistance of *E. coli* cells harboring pLGT2 or a mutant plasmid of it was determined by the agar dilution method and expressed as the minimum inhibitory concentration (MIC).

2.6. Assaying of the reaction of [^{14}C]N-ethylmaleimide (NEM) with TetA(B) proteins

The [^{14}C]NEM-binding experiment was performed as described previously [1].

3. Results

3.1. Random mutagenesis

By means of random mutagenesis as described above, we picked up 1084 colonies resistant to 50 μg/ml kanamycin, from which 47 clones were sensitive to tetracycline (MIC < 50 μg/ml). Among them, 34 clones showed TetA(B) expression (pLGT M1–M34), as judged on Western blotting of the sonicated membranes using anti-Ct14 antiserum (data not shown). Mutation sites which caused a decrease in drug resistance were found to be localized in the C-terminal half (10 clones), N-terminal half (1 clone), upstream region of the *tetA(B)* gene (5 clones), or multiple regions including the C-terminal half (18 clones). Among the 11 mutants of which the mutation sites were only localized in the C- or N-terminal half, six showed tetracycline MICs of higher than 12.5 μg/ml. The other five mutants showed MIC values of less than 1.6 μg/ml and were analyzed in the following experiments. Three of them were found to contain a mutation at codon 332 from GGT (Gly) to AGT (Ser), and two of them contained a double mutation at codons 332 (same as above) and 377 from TGT (Cys) to TAT (Tyr). The MIC values of the G332S single mutant and G332S/C377Y double mutant were 1.6 and 0.8 μg/ml, respectively. In our previous study [18] we showed that Cys-377 is not important for the tetracycline transport function. Thus, the decrease in the drug resistance of these random mutagenesis mutants is mainly due to the mutagenesis at position 332.

3.2. Site-directed mutagenesis at codon 332

In order to confirm the importance of Gly-332 in the TetA(B) function, Gly-332 was replaced with Ser, Ala, Cys, Asp or Leu by site-directed mutagenesis. The mutant proteins were normally expressed in *E. coli* cells, as detected on Western blotting using anti-TetA(B)-C-terminal-peptide antiserum (data not shown). The tetracycline resistance levels of *E. coli* cells expressing these mutant genes are shown in Table 1. G332D and G332L mutants showed no tetracycline resistance. On the other hand, G332S (MIC 1.6 μg/ml) and G332C (6.3 μg/ml) mutants showed very low resistance, and mutant G332A showed moderate resistance (12.5 μg/ml). The MIC values seem to reflect the side chain volume of the residue at position 332. These results are very similar to the results of mutations of Gly-62, which is located at the boundary between cytoplasmic loop2-3 and transmembrane segment II in the N-terminal half of TetA(B). On the other hand, Gly-332 is located at the boundary between cytoplasmic loop 10–11 and transmembrane segment XI in the putative topology of TetA(B) (Fig. 1).

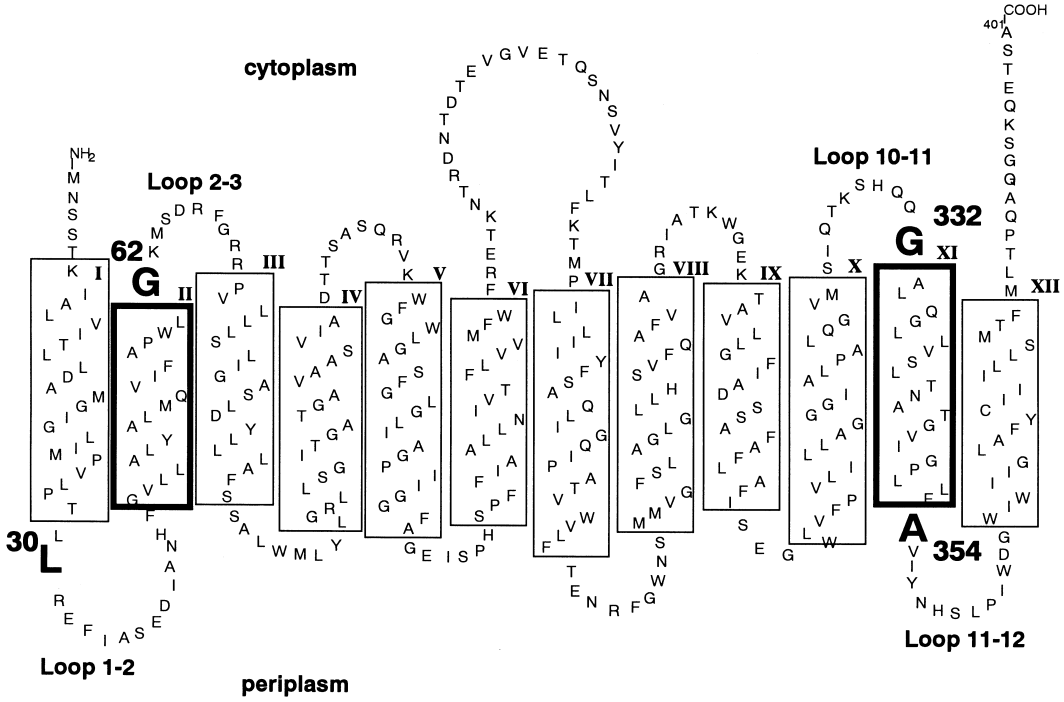

Fig. 1. Putative secondary structure of the TetA(B) protein. The structure was confirmed by site-directed chemical labeling of the Cys mutants of TetA(B) [5]. Large bold letters indicate the important glycine residues described in this manuscript and the residues replaced by the second-site suppressor mutation. Roman numerals indicate the transmembrane helices. Transmembrane helices II and XI are enclosed by bold frames.

Table 1
Tetracycline resistance levels of *E. coli* W3104 cells harboring a wild-type or mutant *tet*A(B) gene

Mutant			Minimum inhibitory concentration (MIC) of tetracycline (μg/ml)
No plasmid			0.8
pLGT2 (wild)			200
pLGT M2 (G332S/C377Y)[a]			0.8
pLGT M22 (G332S)[a]			1.6
	G332S		1.6
	G332A		12.5
	G332C		6.3
	G332D		0.8
	G332L		0.8
pLGT R1 (ΔTT/G332S/A354D)[b]			50
ΔTT			100
ΔTT	G332S		1.6
ΔTT		A354D	100
	G332S	A354D	25
	G332S	A354N	6.3
	G332S	A354G	3.1
	G332S	A354C	1.6
		A354D	100
		A354N	100
		A354G	100
		A354C	100
G332S	L30S		25
G332S	L30S	A354C	12.5
G332S	L30C		6.3
G332S	L30C	A354D	50
G62L			1.6
G62L		A354D	6.3
G62L	L30S		100
G62L	L30C		12.5
G62L	L30C	A354D	50
G62L		A354C	1.6
G62L	L30S	A354D	100

[a]These plasmids were obtained by random mutagenesis. The others except for pLGT R1 plasmid were constructed by site-directed mutagenesis.
[b]This plasmid was obtained from spontaneous mutations of pLGG332S. ΔTT indicates the deletion of two thymidines at position 47 and 48 bases upstream from the first codon of the *tetA(B)* gene.

3.3. Isolation of second-site suppressor mutants from the G332S mutant

In our previous paper [1], we reported that the mutation of Gly-62 is suppressed by a mutation of Leu-30, which is located at the opposite side of the membrane. Thus, we tried to isolate a second-site suppressor mutant from the G332S mutant. 1.0 ml of an *E. coli* cell suspension ($OD_{610} = 1.4$) harboring pLGG332S was applied to an agar plate containing 50 μg/ml tetracycline, followed by incubation at 37°C for 5 days. As a result, 15 colonies were isolated and the plasmids (pLGT R1–R15) were prepared from these revertant cells. The DNA sequences of 14 revertants showed a simple back mutation at codon 332 from AGT (Ser) to GGT (Gly). Only one (pLGT R1) showed a second-site mutation at codon 354 from GCT (Ala) to GAT (Asp). Codon 332 of pLGT R1 was not changed from that of pLG G332S. pLGT R1 had an additional mutation in the palindromic sequence of the promoter OL region, in which two thymidines at positions 47 and 48 bases upstream from the first codon of *TetA(B)* were deleted (ΔTT).

E. coli cells harboring pLGT R1 showed an MIC value of 50 μg/ml; however, the expression of the mutant protein was drastically reduced (data not shown). It was reported by Weissmann et al. [19] that deletion of thymidine in the O_L region of the *tet* operon caused a decrease in the *tetA(B)* gene expression. The ΔTT/G332S mutant without the A354D mutation showed no recovery of tetracycline resistance (Table 1). In contrast, the G332S/A354D double mutant, which was constructed by site-directed mutagenesis, showed normal expression and a MIC value of 25 μg/ml (Table 1), while the expression of this mutant significantly slowed down the cell growth.

Ala-354 is located on the periplasmic surface of the membrane (Fig. 1), thus the suppression must be due to the remote conformational effect of A354D mutation similar to the case of the suppression by the L30S mutation of the G62L mutant. Ala-354 itself is not mandatory for the TetA(B) function because all of the single Ala-354 mutants almost completely retained the wild-type tetracycline resistance level (100 μg/ml) (Table 1). When Ala-354 was replaced with a residue other than Asp in G332S mutants, the resulting double mutant showed moderate or weak recovery of the drug resistance (Table 1).

3.4. Remote conformational change caused by G332S mutation

In the case of G62L mutation, the side chains at positions 29 and 30, which in the wild-type protein are exposed to the periplasmic side, are embedded in the membrane through a remote conformational change, and the suppressor mutation of L30S blocks this conformational change [1]. Therefore, the effect of the G332S mutation on the degree of exposure of the side chain at position 354 was examined. The high reactivity

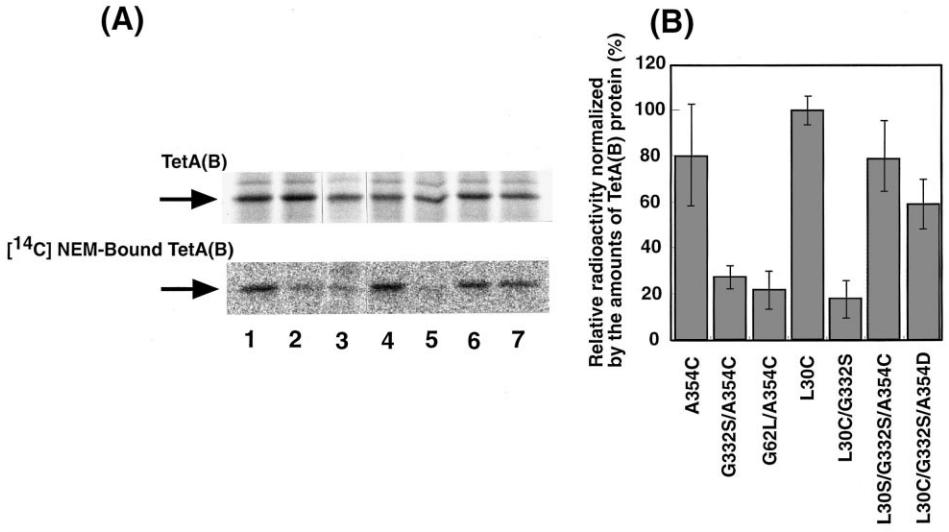

Fig. 2. The binding of [^{14}C]NEM to the mutant TetA(B) proteins. In these experiments, equal amounts of the sonicated membranes (1 mg membrane protein per each sample) were used for incubation with [^{14}C]NEM. After solubilization, the TetA(B) proteins were precipitated with anti-TetA(B) C-terminal antiserum and PANSORBIN [1], followed by SDS-polyacrylamide gel electrophoresis. The expression levels of the mutant TetA(B) proteins were slightly different from each other, therefore, the NEM binding level was normalized by the amount of TetA(B) proteins in panel B. A: Upper panel: Coomassie brilliant blue staining of the SDS-polyacrylamide gel containing the immuno-precipitated TetA(B) proteins. This is a typical result out of three independent experiments. Lane 1: A354C; lane 2: G332S/A354C; lane 3: G62L/A354C; lane 4: L30C; lane 5: L30C/G332S; lane 6: L30S/G332S/A354C; lane 7: L30C/G332S/A354D. Lower panel: Radioactive bands visualized with a BAS-1000 Bio Imaging Analyzer. The lanes are the same as in the upper panel. B: The amount of bound [^{14}C]NEM normalized with respect to the amount of the TetA(B) protein. The data are shown as the average of the results of three independent experiments. The amounts of [^{14}C]NEM and the TetA(B) protein were calculated from the densities of the radioactive bands and Coomassie brilliant blue-stained bands measured with a BAS-1000 Bio-Imaging Analyzer Fuji Film (Tokyo, Japan) and a GS-700 Imaging Densitometer (Bio-Rad), respectively. The values are expressed as percentages of the average level of the L30C mutant values.

of [^{14}C]NEM with the A354C single mutant indicates that the side chain at position 354 is originally exposed to the aqueous phase (Fig. 2). In contrast, the G332S/A354C double mutant hardly reacted with NEM (Fig. 2), certainly indicating that the side chain at position 354 is also buried in the membrane through the remote conformational effect of the G332S mutation, similar to the case of the G62L/L30C double mutant [1].

3.5. Mutual cross conformational suppressions of mutations of Gly-62 and Gly-332

Since the mechanism of A354D suppression of mutant G332S seems very similar to that of mutant G62L suppressed by L30S mutation, we tested the cross suppression of mutants G332S and G62L by L30S and A354D mutations, respectively. Surprisingly, the G332S/L30S double mutant showed a MIC value of 25 μg/ml, which is the same level as the G332S/A354D revertant mutant (Table 1). In the case of the G62L mutant, the A354D mutation caused very low but significant suppression (Table 1). Especially, it should be noted that A354D mutation caused significant a synergistic effect of suppression on the G62L mutant in combination with the L30C mutation (Table 1).

As judged by [^{14}C]NEM binding, position 354 in the A354C mutant was buried into the membrane by the G62L mutation as well as by the G332S mutation (Fig. 2) and position 30 in the L30C mutant was also buried into the membrane by the G332S mutation (Fig. 2) as well as by the G62L mutation [1]. In addition, position 354 in the G332S/A354C double mutant and position 30 in the G332S/L30C double mutant were re-exposed by L30S and A354D suppressor mutations, respectively (Fig. 2). From the MIC values, it is clear that the periplasmic positions 30 and 354 were embedded in the membrane in the inactive form of TetA(B) and exposed to water phase in the active one. These results indicate the presence of close conformational interactions between the regions flanking the transmembrane segments II and XI.

4. Discussion

In the previous study, we observed that absence of bulky side chains on Gly-62 is important for the function of TetA(B) because any mutation of Gly-62 caused the complete loss of the tetracycline export activity [13], except for the Ala mutant. In this study, we found a new glycine residue at position 332 which is important for the TetA(B) function. Similar to Gly-62, the mutations of Gly-332 caused a drastic decrease or complete loss of the tetracycline resistance except for the Ala mutant. The glycine residues of which mutations to glutamic acid residues decreases the tetracycline resistance were also found in loop 10–11 of the pBR322-encoded TetA(C) [20]. Our result indicates that the bulky side chain is deleterious at these glycine positions. Similar role of glycine residues are widely observed in major facilitator superfamily transporters, for example, Jung et al. [21] reported the similar deleterious effect of the bulky side chains at some glycine positions (Gly-64, Gly-115 and Gly-147) in lactose permease. Gly-332 is also conserved in lactose permease, of which a cysteine mutant showed a significant decrease in the lactose transport activity [22].

As reported in our previous paper [1], the mutation of Gly-62 caused a significant conformational change around position 30 which is located on the opposite side of the membrane resulting in the embedding of this area into the membrane. The second-site suppressor mutation at position 30 recovered

the transport function through the blocking of such a remote-conformational change. Similar remote suppression of glycine mutants was also reported in pBR322-encoded TetA(C) [23] and in lactose permease [24]. Jessen-Marshall et al. [24] reported that the mutation of Gly-64 in lactose permease, which corresponds to Gly-62 in TetA(B), is suppressed by a variety of second-site mutations distributed in the mid-transmembrane region and on the periplasmic surface of the membrane. They argued that the loop 2–3 region containing Gly-64 in lactose permease is a conformationally sensitive region which facilitates the conformational change on the opposite side of the membrane [25,26]. Other mutations in TetA(B) in this region were also suppressed by a second-site mutation on the opposite side of the membrane [27]. Therefore, the remote-conformational suppression against mutations in a conformationally sensitive site may be a general rule for major facilitator superfamily transporters [28]. In this study, we found that another remote-conformationally sensitive site is present in the C-terminal half. It is interesting that the mutations at Gly-62 and Gly-332 caused the similar conformational change in the regions around position 30 and 354 located on the periplasmic side of the membrane and both glycine mutants were suppressed by the second-site mutations occurred in either region. These results indicate the presence of a close conformational linkage in these regions and strongly suggested the close proximity of loop 1–2 and loop 11–12 as reported in lactose permease [29–31].

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