Evidence for Interactions between Helices 5 and 8 and a Role for the Interdomain Loop in Tetracycline Resistance Mediated by Hybrid Tet Proteins*

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An interdomain hybrid Tet protein consisting of a class C α domain and a class B β domain (Tet(C/B)) lacks detectable efflux ability and provides only minimal levels of resistance to tetracycline (Tc) (3 μg/ml) compared with intact class B (256 μg/ml) and class C (64 μg/ml). Twenty-one independently isolated mutants of the Tet(C/B) protein with increased Tc resistance were generated by random chemical mutagenesis. Nine mutants with a Glu substitution for Gly-152 in helix 5 of the class C α domain produced a resistance of 48 μg/ml, whereas another 9 with an Asp replacement of Gly-247 in helix 8 of the class B β domain mediated resistance at 32 μg/ml. The third type of mutation, found in 3 mutants expressing 24 μg/ml resistance, was a S202F replacement in the putative interdomain cytoplasmic loop of Tet(C/B). The latter underscores a previously unappreciated function of the interdomain cytoplasmic loop. All three types of Tet(C/B) mutant proteins were expressed in amounts comparable with that of the original protein and demonstrated restored energy-dependent efflux of tetracycline. Site-directed mutational analysis demonstrated that a Gly-247 to Asn mutation could also facilitate Tc resistance by the Tet(C/B) hybrid, and a negatively charged side chain at position 152 was required for Tet(C/B) activity. These mutations appear to promote the necessary functional interactions between the interclass domains that do not occur in the Tet(C/B) hybrid protein and suggest a direct association between helix 5 and helix 8 in the function of Tet efflux proteins.

Tetracycline (Tc)1 resistance is mediated by many different but related determinants in Gram-negative bacteria, designated as classes A through E, G, H, and J (1). Each determinant encodes a cytoplasmic membrane protein, Tet, that catalyzes the active efflux of a tetracycline-divalent cation complex from the cell in exchange for a proton (2). Tet proteins are members of the major facilitator superfamily that includes transporters of various drugs, antibiotics, sugars, and the well-studied lactose permease (3). Members of this group share a common topology as well as regions of amino acid sequence identity.

The Tet proteins of classes B and C are predicted to contain 12 transmembrane (TM) regions divided in half into two domains, α and β, by a large putative cytoplasmic loop designated the interdomain region (4–7). Extensive genetic and mutagenic analyses of the class B Tet protein has shown that the two domains, both of which are required for Tet function (8), contribute differently to the efflux of Tc. In the α domain, amino acid residues in TM2 and TM3 line a putative substrate translocation pathway (9, 10). The cytoplasmic loop connecting TM2 and TM3 could act as a gate that undergoes a conformational change during Tc translocation (11, 12). In the β domain, His-257 in TM8 appears to be involved in proton translocation (13), whereas Asp-285 in TM9 is important for Tc binding (14).

Tc resistance proteins from classes A and C share 78% amino acid sequence identity, whereas the class B determinant is more distantly related to the other two, exhibiting 45% identity (15, 16). A hybrid Tet protein consisting of a class A α domain and class C β domain (Tet(A/C)) specified levels of Tc resistance not unexpected for the combination of the two functional proteins (17). In contrast, a Tet(B/C) protein (class C α domain and class B β domain) provided only minimal resistance to Tc, and a Tet(B/C) protein (class B α domain and class C β domain) did not confer resistance (17). However, an easily detectable level of Tc resistance (10–15% that of the level of wild-type Tet(C)) was observed when both the Tet(C/B) and the Tet(B/C) proteins were expressed together in the same cell. This result indicated that the individual domains of each hybrid protein were active and capable of interacting with the corresponding opposite domain of the same class (18). Presumably, differences between the amino acid sequences of the class C and class B prevented the domains of the Tet(C/B) hybrid from functioning.

In the present study, we have isolated and characterized active Tet(C/B) mutants. The locations of the specific mutations identify areas that probably mediate functional interactions between the α and β domains of Tet proteins and also implicate the interdomain region in the function of the Tet protein.

MATERIALS AND METHODS

Reagents—Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Taq polymerase and T4 DNA ligase were purchased from Life Technologies, Inc. Antibiotics were obtained from Sigma, except that AHTc was prepared by Mark Nelson of this laboratory. [3H]Tc was purchased from NEN Life Science Products. The remaining reagents were analytical grade.

Bacterial Strains and Plasmids—Escherichia coli DH5α cells (recA1; laboratory collection) were used for the propagation of plasmid DNA, determining Tc resistance, and for assessing protein expression. Tc efflux assays were performed with E. coli AG100A cells (ΔacrAB19(19)). Cell cultures were routinely grown in LB media supplemented with chloramphenicol (20 μg/ml) or Tc (10 μg/ml), as needed. AHTc (0.025 μg/ml) was used as a gratuitous inducer of Tet protein where applicable.

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RESULTS

Generation of Tc-resistant Mutants—A total of 21 Tc-resistant mutants were independently isolated from separate mutagenic and transformation events. Sequence analysis of the hybrid tet gene from each mutant revealed three types of mutations, each consisting of a single base change (Table II).

A GGA → GAC transition resulted in the Gly to Glu mutation in nine different mutants. GGC → AAC resulted in the replacement of Gly residue by Asp residue in four different mutants. GGC → GAG resulted in the replacement of Gly residue by Asp residue in three different mutants. A GGC → GAC transition resulted in the Gly to Glu mutation in three different mutants. A GGC → GAG transition resulted in the replacement of Gly residue by Asp residue in two different mutants. A GGC → GCC transition resulted in the replacement of Gly residue by Ala residue in one mutant. A GGC → GCA transition resulted in the replacement of Gly residue by Ala residue in one mutant. A GGC → GCG transition resulted in the replacement of Gly residue by Ala residue in one mutant. A GGC → GGA transition resulted in the replacement of Gly residue by Gly residue in one mutant. A GGC → GGG transition resulted in the replacement of Gly residue by Gly residue in one mutant. 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topological model of Tet(B), the location of Gly-247 is predicted to be within putative helix 8 of the β domain of the Tet(C/B) protein (Fig. 1). Gly at position 247 of Tet(B) is also conserved in all Gram-negative Tet proteins. Cells expressing the G247D Tet(C/B) mutant protein had a Tc MIC of 24 μg/ml, approximately 8-fold over the original Tet(C/B) protein.

Finally, three of the Tet(C/B) mutants isolated contained a UCC to UUC codon change that resulted in a Ser-202 → Phe mutation. A host bearing this mutation exhibited a Tc MIC of 32 μg/ml. Ser-202 is within the Tet(C) α domain of the Tet(C/B) hybrid and resides in the interdomain cytoplasmic loop connecting the α and β domains (Fig. 1).

Each type of mutation was subsequently introduced into the Tet protein from which the corresponding region of the Tet(C/B) protein was derived. Introduction of the G152E mutation into Tet(C/C) (consisting of both an α and a β domain from the class C protein) or the G247D mutation into Tet(B/B) (comprised of class B α and β domains) resulted in inactive proteins, as demonstrated by a Tc MIC of 1.5 μg/ml. A S202F mutation in Tet(C) also led to a severe reduction in Tc resistance to 4 μg/ml from 64 μg/ml mediated by the wild-type protein.

Expression of Parental Versus Mutant Hybrid Tet Proteins—Western blot analysis of the proteins was performed using the anti-Ct antibody directed against the 14 carboxyl-terminal amino acids of Tet(B). The β domain of Tet(C) proteins was altered so that they would be detected by anti-Ct (see “Materials and Methods”). This replacement resulted in a small increase in the Tc MIC provided by these proteins (Table III). Each mutant protein was present in relatively lower amounts.

The parental hybrid Tet(C/B) protein was abundantly present in the membranes of the cells in which it was expressed (Fig. 2A, lane 3). The near background level of Tc susceptibility of Tet(C/B) has been attributed to inefficient functional interactions between α and β domains from the two different classes (17).

The G152E and S202F C/B proteins were detected in the membrane in quantities comparable with the original inactive Tet(C/B) hybrid (Fig. 2A, lanes 3, 4, and 11). The G247D mutant was detected in slightly lower quantities in the membrane compared with the original Tet(C/B) and the other mutant proteins (Fig. 2A, lane 9). Although combining the G152E and G247D mutations in Tet(C/B) did not produce resistance to Tc, the protein was observed in higher quantities in the membrane than the original hybrid or any of the single mutants (Fig. 2B, lane 5).

A strain expressing Tet(B) protein containing the G247D mutation was inactive, which could be attributed to a decreased amount of this mutant protein in the membrane compared with the wild type (Fig. 2B, lane 3). The similar lack of Tc resistance by the Tet(C) G152E could also be due, at least in part, to lower protein expression (Fig. 2C, lane 3). This protein also had a higher mobility in the gel relative to the wild type, which may reflect either a conformational change or the charge difference between the two proteins. It is unlikely, however, to be due to truncation of the G152E protein, as recognition by the anti-Ct antibody requires an intact carboxyl terminus. In addition, a Tet(C) protein containing the G152E mutation complements a deficiency in potassium transport (26), an activity that requires the amino terminus of Tet(C) to be associated with the membrane (27). The presence of the S202F mutation in Tet(C) (generated by site-directed mutagenesis) did not affect the amount of protein in the membrane (Fig. 2C, lane 4). In general, although the Tet(C) proteins analyzed by Western blot were engineered to express the anti-Ct epitope at their carboxyl termini, even the wild-type protein was not as reactive with the antibody as Tet(B) (compare Figs. 2C, lanes 2 and 5). In addition, although Tet(C) with the anti-Ct tag contained only two more amino acid residues, it had a noticeably lower mobility than Tet(B) (Fig. 2C).

Site-directed Mutants—It was of interest to determine whether the negatively charged side chain at either 152 or 247 was a requisite for Tc resistance by the hybrid protein. To this end, G152Q and G247N mutants were generated by site-directed mutagenesis. The Tc MIC of G247N mutant provided a moderate level of Tc resistance (24 μg/ml; Table III). This protein was observed in the membrane in a similar quantity to the wild-type Tet(C/B) hybrid (Fig. 2A, lane 10) and more than the G247D mutant (Fig. 2A, lane 9). Cells expressing the G152Q mutant, however, failed to show the Tet(C/B) protein in the membrane (Fig. 2A, lane 7) and were susceptible to Tc (Table III).

To further investigate the side chain requirements at position 152, additional replacements were constructed. Ala, Asp, and Lys were each substituted for Gly-152; only G152D resulted in a protein capable of mediating a significant level of Tc resistance (10 μg/ml; Table III). Each mutant protein was detectable in the membrane (Fig. 2A), although the G152K protein was present in relatively lower amounts.

These results indicated that an acidic residue was required at position 152 to produce active Tet(C/B). Furthermore, introduction of a neutral or positively charged side chain at position 152 decreased the stability of the Tet(C/B) protein. In contrast, replacing Gly-247 with either Asp or Asn was structurally tolerated and also rendered Tet(C/B) capable of mediating Tc resistance.

Tc Efflux Assays—Energy-dependent Tc efflux was measured as the relative uptake of [3H]Tc before and after deenergization of the cells with the protonophore CCCP. As shown in Fig. 3, AG100A cells not expressing a Tet protein accumulated 26 pmol of [3H]Tc/mg of total protein at 20 min. The addition of 100 μM CCCP resulted in decreased accumulation of Tc in the cell, attributed to the dissipation of the proton gradient across the membrane upon which Tc uptake is dependent. The wild-type class B protein expressed from pLR1068 demonstrated a relatively low uptake of [3H]Tc (9 pmol/mg of protein at 20 min). Deenergization of the cells by CCCP resulted in an increased accumulation of [3H]Tc due to elimination of active Tc efflux. Cells expressing the unmutated Tet(C/B) hybrid from plasmid pRAR1033 did not exhibit efflux activity.

The G152E and G247N Tet(C/B) mutants carried out Tc efflux as well as Tet(B) or Tet(C) (data not shown), accumulating only 8.5 and 9 pmol of [3H]Tc/mg of protein, respectively, at 20 min. Efflux by the G247D and S202F mutants resulted in an accumulation of approximately 11 pmol of [3H]Tc/mg of protein at 20 min. Overall, resistance to Tc mediated by each of the mutant Tet(C/B) proteins was accompanied by energy-dependent efflux of Tc from the cell, although as noted before (8), Tc efflux activity did not directly correlate with the level of Tc resistance.

**Table II**

| Codon change | Number of amino acid substitution | Domain/Location | Tc MIC* μg/ml |
|--------------|-----------------------------------|-----------------|---------------|
| GGA → GAA   | 9 Gly-152 → α domain; helix 5     |                 | 48            |
| GGC → GAC   | 9 Gly-247 → β domain; helix 8     |                 | 32            |
| UCC → UUC   | 3 Ser-202 → Interdomain region    |                 | 24            |

* Determined via E-test. The original wild-type Tet(C/B) protein provided a Tc MIC of 3 μg/ml.
DISCUSSION

Three different single amino acid changes out of a total of 21 independently isolated mutants restored Tc resistance to an otherwise inactive hybrid Tet(C/B) protein. Each mutant demonstrated a MIC for Tc above the 10 μg/ml on which they were selected. Of the $5 \times 10^5$ transformants screened, nine contained a G152E mutation in helix 5 of the class C a domain, and nine more contained the G247D replacement in helix 8 of the class B b domain. This finding suggests that critical interactions between the a and the b domains that are required for the function of Tet(C/B) occur between helices 5 and 8.

Both Gly-152 and Gly-247 are conserved among all classes of Gram-negative Tet proteins and are predicted to be relatively close to the periplasmic side of the membrane (Fig. 1). As conserved amino acid residues are usually of functional or structural significance, it was expected that substitutions leading to an active Tet(C/B) would have been at nonconserved positions. Substitution of Gly-152 with Glu in Tet(C) or Gly-247 with Asp or Asn in Tet(B) had a detrimental effect on Tc resistance, indicating the importance of Gly at these two positions in the wild-type proteins.

Gly-152 is the last residue of an amino acid sequence motif (GXGXGXXGXG) found among the antiporter members of the major facilitator superfamily (28). Our finding that a G152E mutation inactivates Tet(C) agrees with the findings of McNicholas et al. (26). Substitutions at other positions within this conserved motif in Tet(C), specifically Gly-147 (replaced with any other amino acid) (28) or Gly-143 (replaced with Asp or Asn) (26), also reduced or eliminated Tc resistance. Based on molecular modeling of helix 5 of Tet(C), Gly residues within this sequence were concluded to participate in the formation of a substrate binding pocket devoid of side chains (28). If this model holds true, then our result implies that within the Tet(C/B) hybrid, the putative Tc binding site is ineffective, and a G152E mutation suppresses this defect. The fact that only a negatively charged side chain at position 152 could render Tet(C/B) active suggests that an ionic interaction is formed with another region of the protein or that the side chain contributes in some manner to Tc binding. Residues within helix 5 are important for substrate recognition by other major facilitator superfamily members, particularly the multidrug transporter Bmr (29) and the lactose permease (30).

With respect to Gly-247, a helical wheel projection predicts that it is located on the same hydrophilic side of amphipathic helix 8 as Gln-261 and His-257. Gln-261 appears to contribute to substrate recognition by Tet(B) (31), and His-257 has been proposed to play an essential role in proton translocation (13).

With respect to Gly-247, a helical wheel projection predicts that it is located on the same hydrophilic side of amphipathic helix 8 as Gln-261 and His-257. Gln-261 appears to contribute to substrate recognition by Tet(B) (31), and His-257 has been proposed to play an essential role in proton translocation (13).

Introduction of the G247D mutation into Tet(B) apparently

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**FIG. 1.** Predicted topology of the C/B hybrid Tet proteins, based on the respective topological models of the class B (35) and class C (34) Tet proteins. Circled letters indicate the residues that were substituted in Tc-resistant Tet(C/B) mutants; Gly-152 in helix 5 of Tet(C) and Gly-247 in helix 8 of Tet(B) protein are shown. Ser-202 is in the interdomain cytoplasmic loop and the class C half of the hybrid protein. The junction between class C and class B is indicated by an arrow.
TABLE III
Tc resistance mediated by wild-type, hybrid, and mutant hybrid Tet proteins

| Protein | Tc MIC<sup>a</sup> | Protein present |
|---------|------------------|-----------------|
| None    | 1.5              |                 |
| Wild type and hybrid<sup>b</sup> |                 |                 |
| Wild type Tet(B/B) | >256 | 100 |
| Wild type Tet(C/C)<sup>c</sup> | 64 | 100 |
| Wild type Tet(C/B) | 3 | 100 |
| Chemical-generated mutant hybrids |                 |                 |
| Tet(C/B) G152E | 48 | 90 |
| Tet(C/B) G247D | 24 | 59 |
| Tet(C/B) S202F | 32 | 64 |
| Site-directed mutant hybrids |                 |                 |
| Tet(C/B) G152A | 4 | 107 |
| Tet(C/B) G152D | 10 | 95 |
| Tet(C/B) G152Q | 1.5 | -- |
| Tet(C/B) G152K | 5 | 31 |
| Tet(C/B) G247N | 24 | 84 |
| Single-class mutants |                 |                 |
| Tet(C/C) S202F<sup>d</sup> | 4 | 111 |
| Tet(C/C) G152E<sup>d</sup> | 1.5 | 60 |
| Tet(B/B) G247D | 1.5 | 59 |
| Double mutant |                 |                 |
| Tet(C/B) G152E/G247D | 1.5 | 132 |

<sup>a</sup> Determined by E-test.

<sup>b</sup> Proteins were quantitated using the NIH Image 1.6 program available on the Internet. Quantities of mutant Tet(B), Tet(C), and Tet(C/B) proteins are represented as a percentage of the respective wild-type proteins within the same blot. Wild-type proteins of different classes within the same blot were each assigned a value of 100%.

<sup>c</sup> The two letters indicate the class of Tet protein from which the respective α and β domains were derived.

<sup>d</sup> Tc resistance levels for Tet(C) proteins reflect the MIC before the addition of the anti-Ct epitope to the carboxy terminus.

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 **Fig. 2.** Western blot of wild-type, hybrid, and mutant hybrid Tet proteins. Membrane fractions were prepared from DH5α cells expressing various Tet proteins, and 2.5 μg of total membrane protein per lane was loaded, with the exception that membrane containing Tet(C) proteins were loaded at 10 μg/lane. Blotted proteins were probed with an antibody specific for the 14 carboxyl-terminal amino acids of Tet(B). A, lanes 1, no Tet protein; 2, Tet(B/B); 3, Tet(C/B); 4, Tet(C/B) G152E; 5, Tet(C/B) G152A; 6, Tet(C/B) G152D; 7, Tet(C/B) G152Q; 8, Tet(C/B) G152K; 9, Tet(C/B) G247D; 10, Tet(C/B) G247N; 11, Tet(C/B) S202F; B, lanes 1, no Tet protein; 2, Tet(B/B); 3, Tet(B/B) G247D; 4, Tet(C/B); 5, Tet(C/B) G152E/G247D. C, lanes 1, no Tet protein; 2, Tet(C/C) with anti-Ct tag; 3, Tet(C/C) G152E with anti-Ct tag; 4, Tet(C/C) S202F with anti-Ct tag; 5, Tet(B/B).

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**Fig. 3.** Uptake of [3H]-Tc by E. coli AG100A cells expressing wild-type, hybrid, or mutant hybrid Tet proteins. Accumulation was measured as pmol of [3H]-Tc accumulated/mg of protein. Assays were performed at 30 °C with 4 μM [3H]-Tc added after a 5-min preincubation in the presence of 0.2% glucose. CCCP (100 μM) addition is indicated by an arrow.

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