Orientation of the carboxyl terminus of the transposon Tn10-encoded tetracycline resistance protein in *Escherichia coli*

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A site-directed antibody was generated against a synthetic polypeptide corresponding to the 14 amino acid residues of the carboxyl terminus of the Tn10 TetA protein. The antibody reacted preferentially with inside-out vesicles, rather than right-side-out vesicles, prepared from *Escherichia coli* cells harboring transposon Tn10. When inside-out vesicles were treated with trypsin, the TetA protein was completely digested in the vicinity of the carboxyl terminus, as judged on immunoblot analysis using the antibody. In contrast, when right-side-out vesicles were treated with trypsin, the TetA protein was hardly digested. These results indicate that the carboxyl terminus of TetA is exposed to the cytoplasmic side of the membrane.

Tetracycline resistance protein; Tetracycline; Antiporter; TetA; Topology; Site-specific antibody

1. INTRODUCTION

Transposon Tn10 encodes the TetA protein responsible for high-level resistance to tetracycline [1]. The TetA protein is a cytoplasmic membrane protein and acts as a carrier for exclusion of a metal–tetracycline complex out of cells coupled with proton influx [2–4]. The DNA sequence of the *tetA* gene has been determined [5] and, on the basis of its hydropathy profile and its protease digestion pattern, the two-dimensional topology of the TetA protein was proposed by Eckert and Beck [6]. According to their model, the TetA protein is composed of 12 transmembrane segments and, thus, the amino and carboxyl termini are postulated to be located on the same side of the membrane.

Eckert and Beck [6] also suggested that the carboxyl terminus is exposed on the cytoplasmic side, on the basis of the results of carboxypeptidase digestion of spheroplasts and inside-out vesicles prepared from maxicells. However, there remains some ambiguity, because the change in the electrophoretic mobility with carboxypeptidase digestion is very small [6]. In this study, we clearly demonstrate the orientation of the carboxyl terminus by using an immunological approach.

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Abbreviations: RSOV, right-side-out vesicles; ISOV, inside-out vesicles; ELISA, enzyme-linked immunosorbent assay

2. MATERIALS AND METHODS

2.1. *Bacterial strains and growth conditions*

The *E. coli* K12 derivative strains, S3104 ($galT_{12}$, rpoB) [7] and W3104/R388::Tn10 [8], were used in this study as sensitive and resistant strains to tetracycline, respectively. The Tn10-bearing plasmid, R388::Tn10, encodes resistance to trimetoprim and sulfonamide, in addition to that to tetracycline.

These strains were grown in minimal medium [2] supplemented with 0.2% glucose and 0.1% casamino acid, with vigorous shaking at 37°C. In the case of tetracycline-resistant cells, 4 μg/ml of tetracycline was added to the culture as a *tet* gene inducer.

2.2. *Preparation of membrane vesicles*

Inside-out vesicles (ISOV) were prepared from cells lysed with a French pressure cell (1000 kg/cm^2) in 10 mM Tris-Cl (pH 7.5) containing 5 mM $MgCl_2$, and 10 μg/ml DNase I and RNase A [9]. After removal of unbroken cells, the vesicles were washed once with the same buffer, and then stored in 10 mM Tris-Cl (pH 7.4) containing 5 mM $MgSO_4$ and 10% sucrose at −80°C.

Right-side-out vesicles (RSOV) were prepared according to Kaback [10]. The RSOV were finally suspended in 0.1 M potassium phosphate buffer (pH 6.6) and then stored at −80°C.

2.3. *Preparation of an anti-carboxyl terminal antibody*

An oligopeptide corresponding to the putative carboxyl terminal 14 amino acid residues (H_2N-Leu-Thr-Pro-Gln-Ala-Gln-Gly-Ser-Lys-Gln-Glu-Thr-Ser-Ala-COOH) of the Tn10 TetA protein was synthesized with an Applied Biosystems Peptide Synthesizer Model 430A. The peptide, named Ct_{14}, was coupled with bovine thyroglobulin [11]. Rabbit antiserum against the conjugate was prepared using an emulsion of the conjugate with Freund's complete adjuvant (Difco Laboratory). The immunoglobulin (IgG) fraction was precipitated with ammonium sulfate and then purified by affinity chromatography using an immunosorbent prepared by conjugation of Ct_{14} to activated CNBr-Sepharose 4B (Pharmacia).

2.4. *Immunoblotting*

Detection of the TetA protein in the membrane vesicles was performed by Western blot analysis using the anti-Ct_{14} antibody accor-

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ding to the method of Carrasco et al. [11]. After SDS polyacrylamide gel electrophoresis of the membrane vesicles, the proteins were electroblotted onto a nitrocellulose membrane and then TetA was detected as to the binding of the anti-Ct_{14} antibody, followed by the binding of alkaline phosphatase-labeled anti-rabbit IgG (Bio-Rad). The TetA band was stained with an Expressblot kit purchased from Bio-Rad.

2.5. *Competitive ELISA*

Competitive ELISA of ISOV and RSOV using the anti-Ct_{14} antibody was performed by the method of Komeiji et al. [12]. The purified anti-Ct_{14} antibody was biotinylated. Serial dilution of ISOV or RSOV prepared from *E. coli* W3104 or W3104/R388::Tn10 cells containing 0.1% BSA were preincubated with 3 μg/ml of the biotinylated IgG for 1 h at 37°C. Each well of a microtiter plate (Immulon 2; Dinatec. Lab.) was coated with 50 μl of the Ct_{14} peptide (1 μg/ml in PBS, pH 7.4) overnight at 4°C and then blocked by incubation with 3% BSA-PBS for 1 h at 37°C. After washing with PBS, each well was incubated with 50 μl of a mixture of the IgG and vesicles for 1 h at 37°C. The IgG bound to each well was assayed with the avidine–peroxidase conjugate (Vector). The absorbance at 492 nm was monitored with a Chromo Scan ELISA reader (MTP32; Corona).

3. RESULTS AND DISCUSSION

3.1. *Trypsin digestion of RSOV and ISOV*

Both RSOV and ISOV prepared from *E. coli* W3104/R388::Tn10 cells gave the TetA band (36 kDa) specifically detected on immunoblotting using the anti-Ct_{14} antibody (Fig. 1). The vesicles were digested with trypsin before the immunoblot analysis for the indicated periods, the digestion being terminated by the addition of a trypsin inhibitor (Fig. 1). In the case of ISOV, the TetA band had disappeared after 15 min preincubation with trypsin. In the course of the digestion, a band of a smaller fragment of TetA did not appear except for a very thin 21 kDa band, which transiently appeared after 5 min preincubation and then disappeared on longer preincubation, indicating that trypsin primarily digests TetA in the vicinity of its carboxyl terminal epitope. Possible sites for trypsin digestion in the vicinity of the carboxyl terminus are Lys^{327} and Lys^{396}. If trypsin first digests TetA at Lys^{327}, a 0.9 kDa fragment should be detected on immunoblotting. In contrast, if trypsin first digests TetA at Lys^{396}, the epitope should be destroyed and, as a result, the band will disappear. As judged from the results of the immunoblot experiment in Fig. 1, the latter is the case.

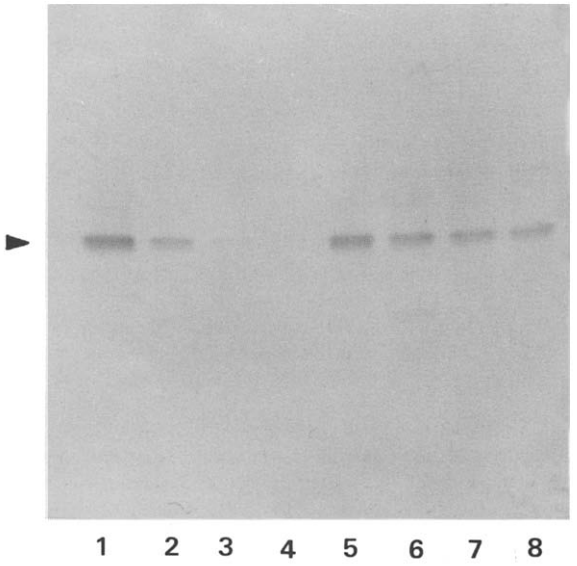

Fig. 1. Immunoblot analysis of trypsin-digested ISOV and RSOV prepared from *E. coli* W3104/R388::Tn10 cells. One mg protein/ml of ISOV or RSOV in 50 mM potassium phosphate buffer (pH 7.0) was incubated with 5 μg/ml trypsin at 37°C for the indicated periods. After the addition of 0.5 μg/ml (final concentration) soybean trypsin inhibitor, the vesicles were precipitated by ultracentrifugation. The samples were electrophoresed on SDS polyacrylamide gels and then the TetA bands were detected by immunoblotting as described in section 2. Lanes 1–4, ISOV; lanes 5–8, RSOV. Lanes 1 and 5, before trypsin digestion; lanes 2 and 6, trypsin digestion for 5 min; lanes 3 and 7, digestion for 15 min; lanes 4 and 8, digestion for 30 min.

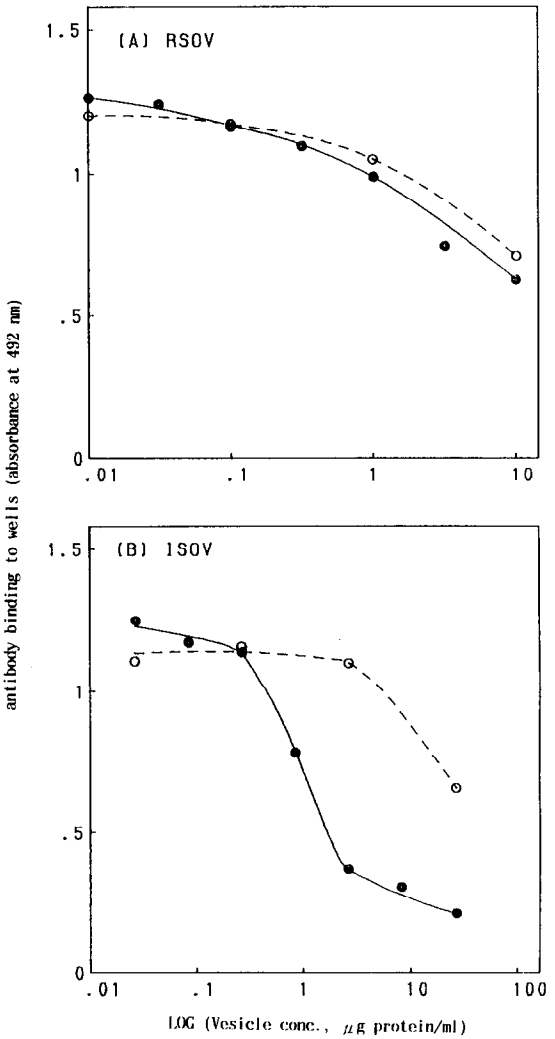

Fig. 2. Ability of vesicles to inhibit antibody binding to the Ct_{14} peptide coated on a plate. Competitive ELISA was carried out by the method of Komeiji et al. [12]. (A) and (B) are for ISOV and RSOV, respectively. Vesicles were prepared from *E. coli* W3104 (○) and W3104/R388::Tn10 (●) cells.

In the case of RSOV, the density of the TetA band at 36 kDa was only slightly decreased. In this case, there was also no band of a smaller fragment except for the thin 21 kDa band after 5 min preincubation, indicating that the TetA protein is not digested from the periplasmic side of the membrane. Considering the putative secondary structure proposed by Eckert and Beck [6], the sizes of the hydrophilic loops located on the periplasmic side are significantly smaller than those on the cytoplasmic side. Although three arginine residues, which are possible sites for trypsin digestion, are assumed to be located on the periplasmic side, trypsin cannot approach these residues, possibly because they are buried due to their small size.

These results indicate that the carboxyl terminus of the TetA protein is located on the cytoplasmic side and that it is primarily degraded through trypsin digestion, if trypsin acts from the cytoplasmic side.

3.2. *Orientation of the carboxyl terminus determined by competitive ELISA*

The accessibility of anti-carboxyl terminal antibodies to membrane proteins was successfully used to determine the location of the carboxyl terminus [11,12]. In this study, the binding of the anti-Ct_{14} antibody to ISOV and RSOV was quantitated by competitive ELISA [12]. As judged on immunoblot analysis, RSOV contained about 2.7-fold more TetA protein than ISOV per mg membrane protein (data not shown). Therefore, as shown in Fig. 2, 2.7-fold more ISOV than RSOV were used for the competitive ELISA. As shown in Fig. 2, ISOV prepared from the resistant strain bound far more antibody than those prepared from the sensitive strain, whereas RSOV from both the resistant and sensitive strains bound almost equal amounts of the antibody. The relatively weak antibody binding to RSOV may represent non-specific binding because, at high vesicle concentrations, minor bands were observed on immunoblot analysis, even for ISOV and RSOV prepared from the sensitive strain (data not shown).

This indicates that the anti-Ct_{14} antibody was only accessible from the cytoplasmic side, which is consistent with the results as to the accessibility of trypsin described above. The results presented here support the orientation of the TetA protein in the model presented by Eckert and Beck [6].

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