Peptide-specific Antibody for the Melibiose Carrier of *Escherichia coli* Localizes the Carboxyl Terminus to the Cytoplasmic Face of the Membrane*

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The synthetic decapeptide NH₂-Cys-Val-Gly-Ala-Val-Ser-Asp-Val-Lys-Ala-COOH (designated MBctlO), which corresponds to the carboxyl terminus of the melibiose carrier of *Escherichia coli*, was synthesized and used to raise antibodies in a rabbit. Anti-MBctlO antibodies recognize the normal melibiose carrier but not a truncated carrier lacking 14 carboxyl-terminal amino acids. Thus the antibodies are specific for the carboxyl terminus of the carrier and not for other domains of the protein. When right-side-out and inside-out membrane vesicles were probed with anti-MBctlO serum, only the inside-out vesicles bound antibody. The carboxyl terminus of the melibiose carrier protein is therefore exposed on the cytoplasmic surface of the membrane. The co-localization of both NH₂- and carboxyl termini to the cytoplasmic surface dictates that the protein cross the membrane an even number of times. These data together with hydrophobicity analysis support a topological model for the melibiose carrier with 10 or 12 transmembrane domains.

The melibiose carrier of *Escherichia coli* is a cytoplasmic membrane protein which mediates the cotransport of a variety of α- and β-galactosides with monovalent cations (see Ref. 1 for a review). Although most bacterial cotransport systems utilize H⁺ gradients (2), the melibiose carrier is unusual in its ability to use H⁺, Na⁺, or even Li⁺ as the coupled cations (3-7). It may thus represent a descendant of the class of transport proteins that bridged the gap between the “H⁺ economy” of primitive cells and the “Na⁺ economy” of animal cells (8, 9).

The gene encoding the melibiose carrier (melB) has been cloned and sequenced by Tsuchiya and his colleagues (10, 11). The protein is deduced to be composed of 469 amino acids and have a molecular mass of 52,215 daltons (11). However, little is known concerning the topology of the carrier within the membrane. Hydrophathy analysis identifies a short hydrophilic NH₂ terminus, 10 strongly hydrophobic segments joined by hydrophilic regions, and a long hydrophilic COOH terminus (11, 12). Tsuchiya has favored a direct interpretation of these data resulting in a topological model with 10 transmembrane domains and cytoplasmic orientations for both the NH₂ and COOH termini (11). Recently, however, it was proposed that the 52-residue-long hydrophobic domain between Arg291 and Ala346 (significantly longer than the nine other hydrophobic domains) may span the membrane twice resulting in 11 transmembrane segments and a periplasmic orientation for the COOH terminus (13). However, there exists no direct evidence in support of either model.

This paper describes the production and characterization of a polyclonal antibody raised against a synthetic decapeptide that corresponds to the hydrophilic COOH terminus of the melibiose carrier. The antibodies bind to the normal melibiose carrier but not to a melibiose carrier lacking 14 COOH-terminal amino acids, indicating that the antisera is specific for the COOH terminus and not for other domains of the protein. Differential probing of right-side-out and inside-out membrane vesicles with the antisera localized the COOH terminus to the cytoplasmic face of the membrane. These data dictate a model with an even number of transmembrane domains suggesting that the 10-domain model is likely to be correct.

**MATERIALS AND METHODS**

**Peptide Synthesis**—The decapeptide NH₂-Cys-Val-Gly-Ala-Val-Ser-Asp-Val-Lys-Ala-COOH (designated MBctlO)1 was synthesized using solid-phase chemistry according to the methods of Houghten (14) by BioSearch, Inc. (San Rafael, CA). The peptide corresponds to the nine COOH-terminal residues of the melibiose carrier plus an additional amino-terminal cysteine residue used for subsequent hapten conjugation. The purity of the peptide was greater than 94% based on amino acid analysis and high pressure liquid chromatography performed by the manufacturer.

**Hapten Conjugation and Antibody Preparation**—Hapten conjugation and antibody preparation were performed by the Berkeley Antibody Company, Inc. (Richmond, CA). The decapeptide was conjugated to keyhole limpet hemocyanin via the NH₂-terminal cysteine residue using the maleimidobenzoyl succinimidem method (15). A single rabbit was immunized via perilymph modal injection with 0.5 mg of conjugated decapeptide emulsified with Freund's complete adjuvant, followed by four intramuscular booster injections of 0.25 mg of antigen emulsified with Freund's incomplete adjuvant at 21-day intervals. Blood samples were taken 11 days after each booster injection and the titer determined via ELISA against unconjugated antigen. The titer was considered adequate (5570-fold dilution of antisera producing half-maximal optical density in the ELISA assay) and the animal exsanguinated on day 96.

**Western Immunoblotting**—To determine the specificity of anti-MBctlO antibodies, inside-out (ISO) membrane vesicles were analyzed by Western immunoblotting (16). Duplicate samples of approximately 150 mg of protein were separated by SDS-PAGE (17) through a 10% polyacrylamide gel. The gel was cut in half and one set of samples stained with Coomassie Brilliant Blue R-250 (18) and the second set electrophoretically transferred to nitrocellulose for immuno-
munoblotting. Nitrocellulose replicas were briefly stained with Ponceau S to mark the position of the molecular weight standards and then completely detainted in distilled water prior to immunoblotting (18). Immunoblotting was performed with a 1000-fold dilution of anti-MBctlO serum in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.25% (w/v) gelatin, and 0.05% (v/v) Tween 20 for 1 h. Protein bands that bound anti-MBctlO were visualized using a horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin staining system (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Filter Immunoblots—Filter immunoblots were performed as described by Lolkema et al. (19). Briefly, whole cells were spotted on nitrocellulose filters, lysed, washed, and exposed to anti-MBctlO at the dilutions indicated in the figure legends. Filters were washed to remove unbound anti-MBctlO, probed with 125I-Protein A (Amersham Corp.), washed to remove unbound 125I-Protein A, air-dried, and radioactivity quantified by γ-counting. To determine nonspecific binding to the nitrocellulose, filters without cells were processed in tandem with experimental filters and the background values subtracted.

Preparation of RSO and ISO Membrane Vesicles—E. coli strain DW1 (ΔmelAB lacI ΔZY; Ref. 20) containing the expression plasmid pKKMB, which encodes the full-length melibiose carrier (21), the plasmid DW1/pKKMB-ctl4, which encodes a melibiose carrier lacking 14 COOH-terminal amino acids (21), or the parent plasmid pKK223-3, which lacks an insert (Pharmacia LKB Biotechnology, Inc.), were grown to early log phase in M63 minimal salts (100 mM potassium phosphate buffer, pH 7.2, 15 mM (NH₄)₂SO₄, 1 mM MgSO₄) containing 1% glycerol, 0.1 mM isopropyl-β-D-thiogalactoside, 100 μg of ampicillin/ml and vitamin B₁, and used to prepare RSO and ISO membrane vesicles. RSO membrane vesicles were prepared according to the procedure of Kaback (22). ISO membrane vesicles were prepared according to the method of Rosen and Tsujiya (23). Membrane vesicles for determining the sidedness of the COOH terminus were used immediately without freezing. ISO vesicles for Western immunoblotting were stored frozen at −80 °C prior to use.

Binding of Antibody to RSO and ISO Membrane Vesicles—Membrane vesicles were diluted with TBS buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mg/ml bovine serum albumin) to a final concentration of 0.1 mg of membrane protein/ml. Aliquots (150 μl, 15 μg of membrane protein) were incubated with a 1500-fold dilution of anti-MBctlO or preimmune serum for 2 h at room temperature with gentle swirling in cellulose propionate Airfuge tubes (Beckman Instruments). The suspensions were then centrifuged at approximately 110,000 × g for 20 min at 4 °C in a Beckman Airfuge and the supernatant carefully decanted. Pellets were resuspended in 150 μl of TBS containing 125I-Protein A (6 kBq/ml; 1.11 GBq/mg Protein A) and incubated for 2 h at room temperature. The suspensions were again centrifuged as above and the supernatant decanted. To remove unbound 125I-Protein A, the pellets were resuspended in 150 μl of TBS, incubated for 20 min, centrifuged, and the supernatant discarded. Radioactivity was quantified by γ-counting after transferring the Airfuge tubes containing the membrane pellets to an appropriate carrier vial.

RESULTS AND DISCUSSION

Specificity of Anti-MBctlO Antibodies—A peptide corresponding to the COOH-terminal nine amino acids of the melibiose carrier was synthesized, coupled to keyhole limpet hemocyanin as a carrier, and used as an antigen to immunize a single rabbit. ELISA performed against the immobilized peptide MBctlO by the Berkeley Antibody Company (see “Materials and Methods”) indicated that the resulting serum contained antibodies directed against the unconjugated peptide (data not shown). To determine if these antibodies recognized the intact melibiose carrier, anti-MBctlO serum was used for Western immunoblotting of SDS-PAGE-separated membrane proteins from (a) DW1/pKKMB, which expresses the full-length melibiose carrier (21), (b) DW1/pKKMB-ctl14, which expresses a truncated melibiose carrier that is missing the COOH-terminal 14 amino acids but still retains the ability to recognize and actively transport melibiose (21), and (c) DW1/pKK223-3 which lacks a meLB insert and therefore does not express the carrier (Fig. 1). Western immunoblots were performed with a 1000-fold dilution of anti-MBctlO, a dilution that resulted in the maximum difference between anti-MbctlO and preimmune serum in filter assays (Fig. 2). Anti-MbctlO reacted with two diffuse bands of nearly identical electrophoro-
rretic mobility. These bands were only present in membranes prepared from cells expressing the full-length melibiose carrier (pKKMB). Corresponding bands were not detected in membranes prepared from cells lacking the melibiose carrier (pKK223-3) or in membranes prepared from cells containing the COOH-truncated carrier (pKKMB-ctl14). This clearly indicates that the anti-MBctl10 serum contains antibodies that are (a) specific for the melibiose carrier and (b) recognize the COOH terminus of the carrier and not other domains of the protein. The unpurified anti-MBctl10 serum also reacted with two high molecular weight proteins from cells containing pKKMB as well as the control cells lacking the melibiose carrier. Preimmune serum similarly reacted with these two bands (data not shown).

Apparent Molecular Mass of the Melibiose Carrier—The labeled protein exhibits an electrophoretic mobility corresponding to a molecular mass of approximately 37-38 kDa, in close agreement with the 38-40 kDa previously estimated for the melibiose carrier. That the observed electrophoretic mobility is substantially less than the predicted molecular mass of 52,215 Da (15) is a common feature of many integral membrane proteins. For example, the lactose carrier has a predicted molecular weight of 46,500 (24) but exhibits an apparent molecular mass of 30 kDa by SDS-PAGE (25). Such discrepancies are presumably a product of the strongly hydrophobic nature of integral membrane proteins which results in the binding of a proportionally greater number of SDS molecules and thus a greater net negative charge/unit length of protein. Since molecular weights are estimated from SDS-PAGE under the assumption of uniform charge/unit length of protein, hydrophobic proteins will migrate proportionally faster per unit length than hydrophilic proteins resulting in the observed molecular weight discrepancies.

Careful examination of the labeled band indicates that it is actually composed of a doublet of bands with less than 1000 Da difference between their molecular masses. Both bands are detectable only in blots from cells expressing the full-length melibiose carrier. This suggests that both are products of the melB gene and that both have intact COOH termini. Although extreme caution was taken to prevent oxidation prior to and during electrophoresis, all Westerns exhibited the same doublet. This suggests that it is not an artifact of the sample preparation but may reflect some post-translational processing of the carrier. Although it is believed that inner membrane proteins of Gram-negative bacteria do not possess cleavable signal sequences (26, 27), and in particular that the melibiose carrier does not possess a cleavable signal sequence (11), the NH2 terminus of the mature protein has never been directly sequenced to confirm identity with that predicted from the nucleotide sequence. The doublet may thus reflect mature and immature carrier species that differ at their NH2 termini. Direct peptide sequencing would provide further information on the nature of the modification.

Determination of Anti-MBctl10 Specificity: Filter Immunoblot Analysis of Lysed Cells—Lolkema et al. (19) have recently described a simple rapid filter immunoassay for the estimation of lactose carrier (lacY) expression in E. coli. In this procedure, whole cells are simply spotted on nitrocellulose filters, lysed, washed and probed with primary antibody followed by [125I]-Protein A, obviating the need for SDS-PAGE. To assess the usefulness of this filter immunoassay with respect to the melibiose carrier system, a similar experiment to that described above was performed on whole cells and compared to the results obtained from the Western immunoblot analysis.

To determine the appropriate serum dilution for the filter assay, DW1/pKKMB (which expresses the full-length melibiose carrier) was probed with anti-MBctl10 or preimmune serum at 100-, 400-, 1,600-, 6,400-, 25,600- and 102,400-fold dilutions (Fig. 2). Dilution between 500- and 2,000-fold yielded the maximum difference between anti-MBctl10 and preimmune sera. All subsequent filter immunomassays were therefore performed at a 1,600-fold serum dilution.

Filter immunoanalysis of cells expressing the full-length or truncated melibiose carrier and cells lacking the melibiose carrier mirrored the results of Western analysis (Fig. 3). Anti-MBctl10 reacted strongly only with cells expressing the full-length carrier. These results indicate that the filter assay may be used in place of SDS-PAGE to estimate the expression of
the melibiose carrier as long as appropriate controls for the host background and linearity of response are performed.

Localization of the COOH Terminus of the Melibiose Carrier—Hydrophobicity analysis of the melibiose carrier suggests two orthogonal models for membrane topology. A model with 10 transmembrane domains resulting in the cytoplasmic orientation of both the NH2 and COOH termini (11), or a model with 11 transmembrane domains resulting in the cytoplasmic orientation of the NH2 terminus and a periplasmic localization of the COOH terminus ((13) Fig. 4).3 The differences between the two models are a result of opposing interpretation of the 52-residue-long hydrophobic domain between the COOH terminus in membrane proteins (24, 25).

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REFERENCES
1. Wilson, T. H., Ottina, K., and Wilson, D. M. (1982) in Membranes and Transport (Martonosi, A. N., ed) pp. 33–39, Plenum Publishing Corp., New York
2. Rosen, B. F., and Kashket, E. R. (1978) in Bacterial Transport (Rosen, B. F., ed) pp. 559–620, Marcel Dekker, Inc., New York
3. Tsuchiya, T., Raven, J., and Wilson, T. H. (1977) Biochem. Biophys. Res. Commun. 76, 26–31
4. Tsuchiya, T., Lopilato, J., and Wilson, T. H. (1978) J. Membr. Biol. 42, 45–59
5. Tsuchiya, T., and Wilson, T. H. (1978) Membr. Biochem. 2, 63–70
6. Lopilato, J., Tsuchiya, T., and Wilson, T. H. (1978) J. Bacteriol. 134, 147–156
7. Cohn, D. E., and Kaback, H. R. (1980) Biochemistry 19, 4237–4243
8. Niiya, S., Yamasaki, K., Wilson, T. H., and Tsuchiya, T. (1982) J. Biol. Chem. 257, 8902–8906
9. Wilson, T. H., and Lin, C. C. (1980) J. Supramol. Struct. 13, 421–446
10. Hanatani, M., Yazu, H., Shiota-Niya, S., Moriyama, Y., Kanazawa, H., Futai, M., and Tsuchiya, T. (1984) J. Biol. Chem. 259, 1807–1812
11. Yazu, H., Shiota-Niya, S., Shinzamoto, T., Kanazawa, H., Futai, M., and Tsuchiya, T. (1984) J. Biol. Chem. 259, 4320–4326
12. Kawakami, T., Akiyama, Y., Ishikawa, T., Shunzamoto, T., Tsuchiya, T., and Tsuchiya, T. (1987) J. Biol. Chem. 263, 4243–4250
13. Botfield, M. C., and Wilson, T. H. (1988) J. Biol. Chem. 263, 12909–12915
14. Houghton, R. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5131–5135
15. Liu, F.-T., Zinnecker, M., Hamaoka, T., and Katz, D. H. (1979) Biochemistry 18, 690–697
16. Burnette, W. N. (1981) Anal. Biochem. 112, 195–203
17. Laemmli, U. K. (1970) Nature 227, 680–685
18. Sasse, J. (1987) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 10.6.1–10.6.3, John Wiley & Sons, New York
19. Lokkema, J. S., Puttner, I. B., and Kaback, H. R. (1988) Biochemistry 27, 8307–8310
20. Wilson, D. M., and Wilson, T. H. (1987) Biochim. Biophys. Acta 904, 191–200
21. Botfield, M. B., and Wilson, T. H. (1989) J. Biol. Chem. 264, in press
22. Kaback, H. R. (1971) Methods Enzymol. 22, 99–120
23. Rosen, B. F., and Tsuchiya, T. (1979) Methods Enzymol. 56, 233–241
24. Buchel, D. E., Gronenborn, B., and Muller-Hill, B. (1980) Nature 283, 541–545
25. Jones, T. H. D., and Kennedy, E. (1989) J. Biol. Chem. 264, 5981–5987
26. Owen, P., and Kaback, H. R. (1979) Biochemistry 18, 1422–1425
27. Engelman, D. M., and Steitz, T. A. (1981) Cell 25, 411–422
28. Von Heijne, G. (1981) Eur. J. Biochem. 116, 419–422

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3 Both models assume a cytoplasmic orientation for the NH2 terminus. This is based upon the lack of a cleavable signal sequence (1) which is believed to dictate cytoplasmic localization of the NH2 terminus in membrane proteins (24, 25).