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Binding of monoclonal antibody 4B1 to homologs of the lactose permease of *Escherichia coli*

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Abstract

The conformationally sensitive epitope for monoclonal antibody (mAb) 4B1, which uncouples lactose from H^+ translocation in the lactose permease of *Escherichia coli*, is localized in the periplasmic loop between helices VII and VIII (loop VII/VIII) on one face of a short helical segment (Sun J, et al., 1996, *Biochemistry 35*:990–998). Comparison of sequences in the region corresponding to loop VII/VIII in members of Cluster 5 of the Major Facilitator Superfamily (MFS), which includes five homologous oligosaccharide/H^+ symporters, reveals interesting variations. 4B1 binds to the *Citrobacter freundii* lactose permease or *E. coli* raffinose permease with resultant inhibition of transport activity. Because *E. coli* raffinose permease contains a Pro residue at position 254 rather than Gly, it is unlikely that the mAb recognizes the peptide backbone at this position. Consistently, *E. coli* lactose permease with Pro in place of Gly254 also binds 4B1. In contrast, 4B1 binding is not observed with either *Klebsiella pneumoniae* lactose permease or *E. coli* sucrose permease. When the epitope is transferred from *E. coli* lactose permease (residues 245–259) to the sucrose permease, the modified protein binds 4B1, but the mAb has no significant effect on sucrose transport. The studies provide further evidence that the 4B1 epitope is restricted to loop VII/VIII, and that 4B1 binding induces a highly specific conformational change that uncouples substrate and H^+ translocation.

Keywords: bioenergetics; conformational epitope; oligosaccharide/H^+; symporters; transport

The lactose (lac) permease of *Escherichia coli* is a paradigm for secondary transport proteins from archaea to the mammalian central nervous system (reviewed in Kaback, 1983, 1989, 1992, 1996; Poolman & Konings, 1993). All available evidence indicates that the permease is composed of 12 α-helical rods that traverse the membrane in zigzag fashion with the N- and C-termini on the cytoplasmic face (Fig. 1).

Based on site-directed excimer fluorescence (Jung et al., 1993), site-directed mutagenesis, second-site suppressor, and chemical rescue studies (King et al., 1991; Lee et al., 1992; Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993; Frillingos & Kaback, 1996a; see Lee et al., 1996, in addition), a helix packing model of the C-terminal half of the permease was formulated. The model has been confirmed and extended by engineering divalent metal-binding sites (Jung et al., 1995; He et al., 1995a, 1995b), site-directed chemical cleavage (Wu et al., 1995), site-directed spin labeling and thiol crosslinking (Wu et al., 1996; Wu & Kaback, 1996), and by the demonstration that monoclonal antibody 4B11 binds to the last two cytoplasmic loops (Sun et al., 1997).

Another mAb designated 4B1 blocks deprotonation of the permease and uncouples lactose and H^+ translocation by binding to a conformational epitope on periplasmic surface of lac permease (Carrasco et al., 1984a; Herzlinger et al., 1984). Further studies (Sun et al., 1996) demonstrate that the 4B1 epitope is located in the periplasmic loop VII/VIII on one face of a short α-helical segment. Thus, Cys-scanning mutagenesis demonstrates that Phe247 is the primary epitope determinant, and sulfhydryl modification of single-Cys mutants shows that Phe250 and Gly254 are also important.

Although 4B1 binding uncouples lactose from H^+ translocation, none of the residues in loop VII/VIII is important for activity (Frillingos et al., 1994; Sun et al., 1996). On the other hand, mAb 4B1 decreases the apparent pK_a of an Asp residue in place of Glu325 (Frillingos & Kaback, 1996b), and alters the reactivity of single Cys-replacement mutants in the C-terminal half of the permease [e.g., V238C (helix VII), V331C (helix X), and single Cys 355 (helix XI)] with N-ethylmaleimide (Frillingos et al., 1997). These results and the observation that avidin binding to a biotin-

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Abbreviations: BSA, bovine serum albumin; C-less permease, functional *E. coli* lactose permease devoid of Cys residues; *E. coli* sucrose permease/loop VII/VIII, *E. coli* sucrose permease with loop VII/VIII from the *E. coli* lac permease; EDTA, ethylenediaminetetracetic acid; IPTG, isopropyl 1-thio-β,D-galactopyranoside; KP_i, potassium phosphate; lac, lactose; mAb, monoclonal antibody; MFS, Major Facilitator Superfamily; PMS, phenazine methosulfate; RSO, right-side-out.

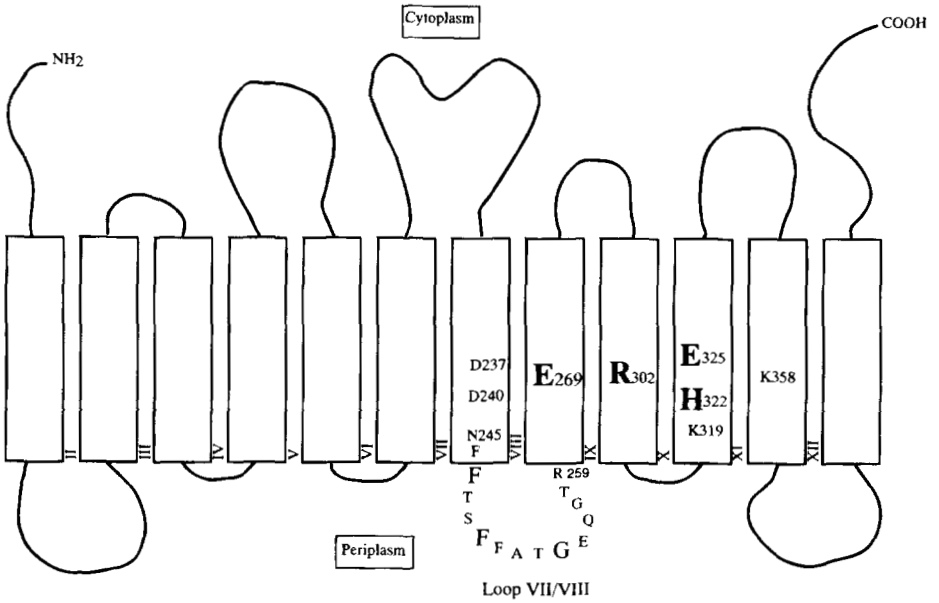

Fig. 1. Secondary structure model of *E. coli* lac permease. The 12 hydrophobic transmembrane helices are depicted as rectangles. The sequence of loop VII/VIII is indicated and the single-letter amino acid code is used. The irreplaceable residues in the permease are emboldened. Two charge pairs (D237 with K358 and D240 with K319) in transmembrane helices VII, X, and XI are indicated.

ylated Cys residue in loop VII/VIII has no effect on transport (Sun et al., 1996) suggest that uncoupling by 4B1 is due to a torsional effect induced by binding, which alters the pK_a(s) of residues that play a direct role in the mechanism.

In this study, 4B1 binding and its effect on activity are examined with the members of Cluster 5 of the Major Facilitator Superfamily (Marger & Saier, 1993; Lee et al., 1994). The sequence of loop VII/VIII (residues 245–259) of the *E. coli* lac permease shares varying degrees of homology with each of the four related symporters, ranging from 100% identity with the *C. freundii* lac permease to 33% identity with the *E. coli* sucrose permease (Table 1). The mAb binds to the *C. freundii* lac permease or the *E. coli* raffinose permease and inhibits transport activity. In contrast, 4B1 neither binds nor inhibits transport with the *K. pneumoniae* lac permease or the *E. coli* sucrose permease. When the epitope is transferred from *E. coli* lac permease to the sucrose permease, 4B1 binding is observed, but the mAb has no effect on activity.

Table 1. *Comparison of the sequence of loop VII/VIII (from Asn245 to Arg259) of* E. coli *lac permease with the corresponding regions of lac permease from* C. freundii *and* K. pneumoniae *M5a1,* E. coli *raffinose permease, and* E. coli *sucrose permease*[a]

Transporter	Sequence (from Asn245 to Arg259 in *E. coli* lac permease)
	* * *
E. coli lacY	N FFTSF FATGE QGTR
C. freundii lacY	N FFTSF FATGE QGTR
K. pneumoniae lacY	N FFkgF Fsspq rGTe
E. coli rafB	i FFeSF FrTpq aGik
E. coli cscB	v Fyagl Feshd vGTR

[a]One-letter amino acid code is used. The main epitope residues in *E. coli* lacY are marked with asterisk. Residues identical to those in *E. coli* lac permease are capitalized.

Results

mAb 4B1 binding

Because 4B1 binds to an epitope in loop VII/VIII of lac permease that shares varying degrees of homology with the corresponding region in five related oligosaccharide/H^+ transporters in MFS Cluster 5 (see Table 1), the homologs were examined for mAb 4B1 binding. Spheroplasts prepared from IPTG-induced *C. freundii* bind 4B1 in a manner comparable to that of spheroplasts containing *E. coli* lac permease (Fig. 2A). Spheroplasts with *E. coli* raffinose permease bind to a lesser extent, and no 4B1 binding whatsoever is observed with spheroplasts containing *K. pneumoniae* lac permease or *E. coli* sucrose permease (Fig. 4A).

Although the sequence of loop VII/VIII in *C. freundii* lac permease is identical to that of *E. coli* lac permease and 4B1 binding is anticipated, only about 50% identity is observed in the loop between *E. coli* lac permease and either *E. coli* raffinose permease, which binds 4B1, or *K. pneumoniae* lac permease, which does not (Table 1; Fig. 2A). In particular, although two of the epitope determinants in *E. coli* lac permease (Phe247 and Phe250) are conserved, Gly254 is replaced with Pro in both cases. Mutant G254P in *E. coli* lac permease was constructed, found to be expressed at control levels in the membrane and binds mAb 4B1 about 45% as well as C-less permease (Fig. 2B). Two residues (Ser249 and Thr253) in the sequence containing the epitope are conserved in raffinose permease, but not in *K. pneumoniae* lac permease where Ser249 is replaced with Gly and Thr253 is replaced with Ser. To

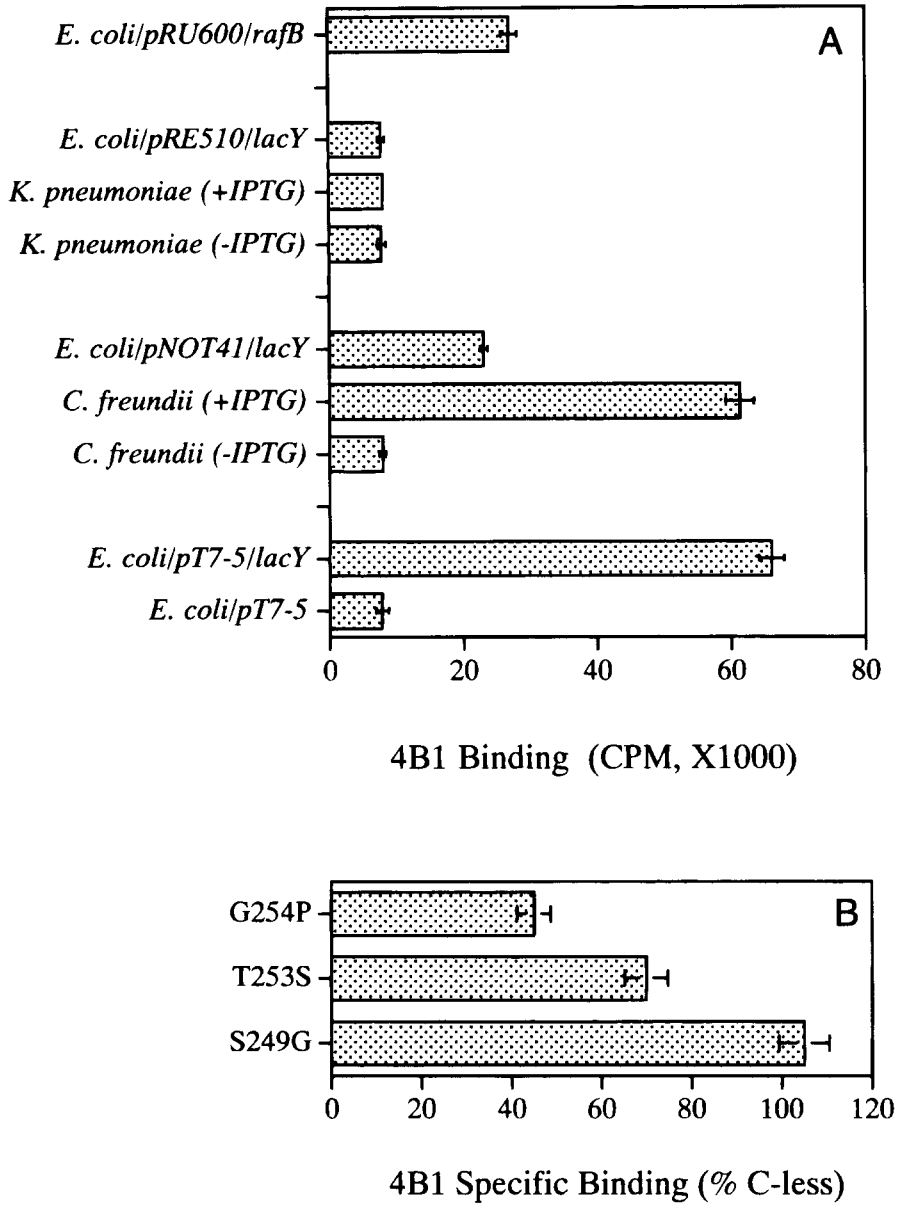

Fig. 2. Binding of mAb 4B1 to homologs of *E. coli* lac permease and its mutants. **A:** Binding to *E. coli* T184 spheroplasts harboring given plasmids or to spheroplasts of *C. freundii* or *K. pneumoniae* M5a1. *E. coli* T184 cells were grown and induced with IPTG. *C. freundii* and *K. pneumoniae* M5a1 were grown in the absence or presence of 0.5 mM IPTG, as indicated. Spheroplasts were prepared and incubated with 4B1 followed by [^{125}I]protein A as described in Materials and methods. 4B1 binding is expressed as radioactivity ([^{125}I]protein A) bound to spheroplast preparations containing equal amounts of protein. The amount of permease expressed in every preparation could not be quantitated due to the lack of appropriate antibodies against *C. freundii* and *K. pneumoniae* lac permeases or the *E. coli* raffinose permease. However, the negative controls (−IPTG) bind only about 11% of positive control (+IPTG). The four groups represent spheroplasts expressing *E. coli* rafB, *K. pneumoniae*, *C. freundii*, and *E. coli* lacY, respectively, and the negative controls. The *K. pneumoniae* and *C. freundii* permeases were also expressed in *E. coli* (*E. coli/pRE510/lacY* and *E. coli/pNOT41/lacY*, respectively). **B:** Binding to spheroplasts expressing mutants of *E. coli* lac permease. The amount of each mutant was quantitated as described in Materials and methods, and 4B1 specific binding is expressed as a percentage of that observed with C-less lac permease. All data were corrected for 4B1 binding to spheroplasts harboring plasmid pT7-5 without a *lacY* insert. The results represent the average of two independent experiments.

examine the role of the two residues in more detail, mutants S249G and T253S were constructed in *E. coli* lac permease (Fig. 2B). Both mutants are expressed in the membrane to control levels, and S249G binds 4B1 about as well as the control, while T253S binds about 70% as well. Finally, only 31% identity is observed between *E. coli* lac permease and *E. coli* sucrose permease, and no conservation of Phe247, Phe250, or Gly254, which comprise the epitope (Sun et al., 1996) is observed in sucrose permease (Table 1). Therefore, the finding that sucrose permease does not bind 4B1 is expected.

Active transport

Active transport catalyzed by *E. coli* lac permease is markedly inhibited as a result of 4B1 binding (Carrasco et al., 1984a; Sun et al., 1996), and the four irreplaceable residues in *E. coli* lac permease (Glu269, Arg302, His322, and Glu325) are conserved in the homologous symporters. Therefore, the effect of 4B1 on active transport was tested in the homologous members of Cluster 5. 4B1 inhibits active transport of either melibiose (Fig. 3A) or lactose (not shown) by *C. freundii* lac permease in RSO membrane vesicles to an extent comparable to that observed with *E. coli* lac permease (ca. 80%). In addition, mAb 4B1 inhibits active transport of either raffinose (Fig. 3B) or melibiose (not shown) by *E. coli* raffinose permease, but to a lesser extent (ca. 50%), a finding consistent with the observation that 4B1 binding to raffinose permease is decreased relative to *E. coli* lac permease or *C. freundii* lac permease (Fig. 2A). As expected, no inhibition of transport by 4B1 is observed with *K. pneumoniae* lac permease or *E. coli* sucrose permease, neither of which binds the mAb (data not shown).

Grafting the 4B1 epitope into the sucrose permease

To examine whether the mAb 4B1 epitope can be transferred from *E. coli* lac permease to a homolog that does not bind the mAb, loop VII/VIII in *E. coli* sucrose permease was replaced with the corresponding region (residues 245–259) of *E. coli* lac permease to construct *E. coli* sucrose permease/loop VII/VIII. In addition, sucrose permease/loop VII/VIII was engineered to contain the C-terminal dodecapeptide of *E. coli* lac permease to allow quantitation by Western blotting (Frillingos et al., 1995; Sahin-Tóth et al., 1995). *E. coli* sucrose permease/loop VII/VIII is expressed in the membrane about 50% as well as wild-type sucrose permease and catalyzes sucrose transport. Relative to *E. coli* lac permease, the engineered sucrose permease mutant, binds 4B1 about 70% as well (Fig. 4A). However, 4B1 binding does not inhibit sucrose transport in RSO membrane vesicles containing chimeric sucrose permease to any significant extent (Fig. 4B).

Discussion

The studies reported here provide further characterization of the epitope for mAb 4B1, which binds to loop VII/VIII of the *E. coli* lac permease and uncouples lactose from H^+ translocation. The *C. freundii* lac permease exhibits 70% identity with *E. coli* lac permease and binds 4B1. Moreover, activity is inhibited by 4B1 to approximately the same extent as observed with *E. coli* lac permease. Similarly, *E. coli* raffinose permease has 56% identity with *E. coli* lac permease and also binds 4B1, but both binding and the inhibitory effect of the mAb are appropriately decreased relative to *E. coli* lac permease. In contrast, neither the *K. pneumoniae* lac permease nor the *E. coli* sucrose permease binds 4B1 nor is the mAb inhibitory, although the two transporters exhibit about 60% and 31% identity, respectively, with *E. coli* lac permease. Finally, when the epitope is transferred from *E. coli* lac permease to the sucrose permease, although 4B1 binding is observed, the mAb has no significant effect on activity.

As shown by Sun et al. (1996), Phe247, Phe250, and Gly254 comprise the 4B1 epitope, and these residues probably fall on one face of a short helical segment. Furthermore, thiol modification studies on single-Cys replacement mutants in the short helical segment show that modification of certain Cys residues on the

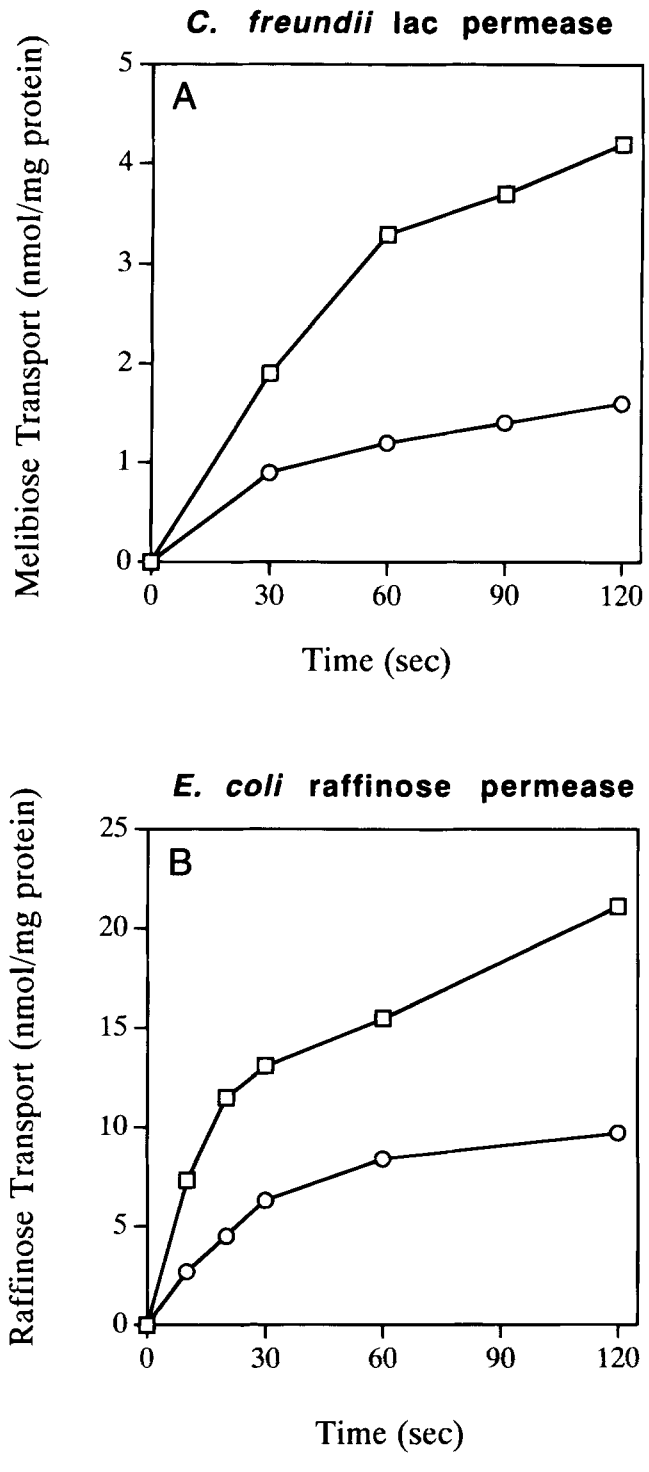

Fig. 3. Effect of mAb 4B1 on active transport of *C. freundii* lac permease and *E. coli* raffinose permease. RSO vesicles from *C. freundii* or *E. coli* T184 expressing *E. coli* raffinose permease were prepared and assayed either with no further treatment or incubated at 25 °C for 30 min with 0.7 mg/mL of 4B1. [^{3}H]Melibiose or [^{3}H]raffinose transport in the presence of ascorbate and PMS was assayed under oxygen as described in Materials and methods. **A:** Effect of mAb 4B1 on melibiose transport in RSO membrane vesicles from induced *C. freundii*. Open square, no additions; open circle, 4B1 added. **B:** Effect of mAb 4B1 on raffinose transport in RSO membrane vesicles from *E. coli* T184 harboring plasmid with the *E. coli* rafB gene. Open square, no additions; open circle, 4B1 added.

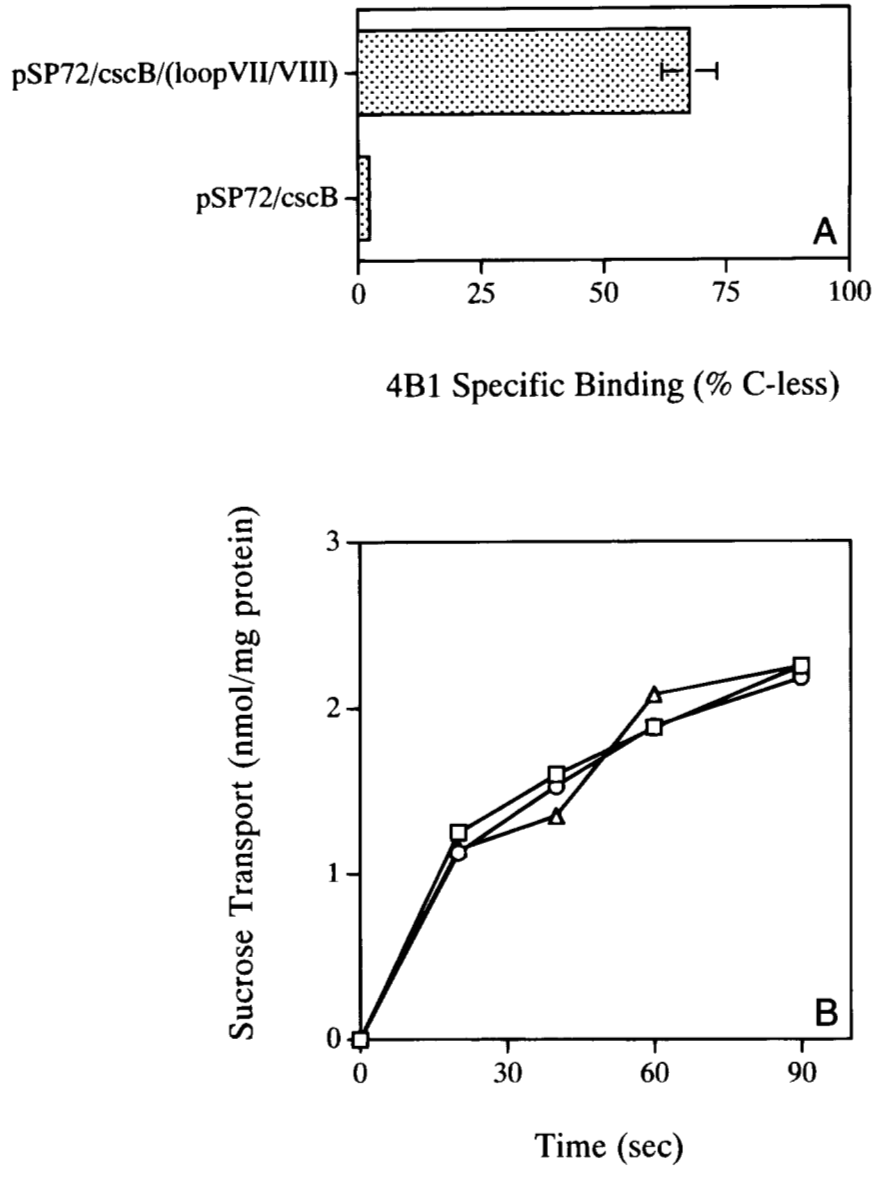

Fig. 4. A: Binding of mAb 4B1 to *E. coli* T184 spheroplasts expressing *E. coli* sucrose permease/loop VII/VIII. Cells were grown and induced with 0.5 mM IPTG. Spheroplasts were prepared and incubated with 4B1 and [^{125}I]protein A as described in Materials and methods. The amount of permease expressed in each instance was quantitated by immunoblotting. **B:** Effect of mAb 4B1 on sucrose transport by *E. coli* sucrose permease/loop VII/VIII. RSO vesicles were prepared, and [^{14}C(U)]sucrose transport was assayed in the presence of ascorbate and PMS under oxygen as described in Materials and methods. Open square, no additions; open circle, 2 mg/mL 4B1 added; open triangle, 4 mg/mL 4B1 added.

opposite face leads to increased 4B1 binding. Because a Gly residue is found at position 254, it was postulated that in addition to two helical turns, the epitope might contain a β-turn. *E. coli* raffinose permease exhibits about 50% identity within loop VII/VIII and also binds 4B1 about 50% as well as *E. coli* lac permease. More specifically, Phe247 and Phe250 are conserved, but Gly254 is replaced with a Pro residue. In addition, 4B1 binding by G254P *E. coli* lac permease is comparable to that observed with *E coli* raffinose permease. Taken as a whole, the results are consistent with the argument that 4B1 recognizes a structural feature at position 254 rather than the peptide backbone, although it cannot be stated with certainty that there is a β-turn at this position (Wilmot & Thornton, 1988). It is also noteworthy that Thr248 and Ala252, which are on the opposite face of the putative short helix, are replaced with Glu and Arg, respectively, in *E. coli* raffinose permease.

Although Phe247 and Phe250 are conserved in *K. pneumoniae* lac permease, there are differences in five residues in the epitope relative to *E. coli* lac permease: Thr248, Ser249, Ala252, Thr253, and Gly254 are replaced with Lys, Gly, Ser, Ser, and Pro, respectively (Table 1). *E. coli* raffinose permease, which binds 4B1, contains Glu248 and Arg252. Thus, position 248 or 252 cannot play an essential role in 4B1 recognition. S249G *E. coli* lac permease binds 4B1 as well as control, the T253S mutant binds about 70% as well, and mutant G254P binds about 50% as well.

Therefore, it is apparent that a change in each of these three residues is insufficient for abrogation of 4B1 recognition and that it is the combined effect of the alterations that is probably responsible.

The *E. coli* sucrose permease, which exhibits only 31% identity with *E. coli* lac permease, does not bind 4B1 nor does the mAb inhibit activity. Clearly, this can be attributed to the lack of conservation of the residues that determine 4B1 recognition (i.e., Phe247, Phe250, and Gly254). The only residues in the epitope that are conserved in sucrose permease are Phe246 and Phe251 (Table 1). Remarkably, however, when residues 245–259 from *E. coli* lac permease are transferred into the corresponding region of sucrose permease, the chimera binds 4B1 about 70% as well as lac permease. Therefore, the 4B1 recognition site is clearly restricted to this sequence of amino acid residues in loop VII/VIII of lac permease. On the other hand, binding of 4B1 to sucrose permease/loop VII/VIII has no significant effect on activity.

Although three of the four irreplaceable residues in *E. coli* lac permease [Arg302 (helix IX), His322 (helix X) and Glu325(helix X)], as well as a functionally interacting charge pair [Asp240 (helix VII) and Lys319 (helix X)], are conserved in the members of Cluster 5, Glu269 is not conserved in *E. coli* sucrose permease. Rather, a Val residue is in the corresponding position in helix VIII of sucrose permease, and a Glu residue is at position 272 (Bockmann et al., 1992) (i.e., on the same face of helix VIII as 269 but one turn removed toward the cytoplasmic face of the membrane). Furthermore, Asp237 (helix VII) and Lys358 (helix XI) in lac permease are replaced with Asn234 and Ser356, respectively, in sucrose permease. When an Asp and a Lys residue are introduced into sucrose permease at positions 234 and 356, respectively, the double mutant exhibits high activity. However, unlike the situation in *E. coli* lac permease where reversal of Asp237 and Lys358 has little effect on activity (Dunten et al., 1993), reversal of the charge pair in sucrose permease double mutant abolishes activity (Frillingos et al., 1995). Therefore, although the lac and sucrose permeases are related and appear to need an Asp-Lys salt bridge between helices VII and XI for optimal insertion into the membrane (Frillingos et al., 1995), the two proteins exhibit distinct differences.

Materials and methods

E. coli T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$*(A), rspL, met*$^-$*, thr*$^-$*, recA, hsdM, hsdR/F', lac I*^{q}*O*$^+$*Z*^{D118}*(Y*$^+$*A*$^+$*)*] (Teather et al., 1980) was routinely used for transformation. Other cell strains and plasmids are described in Table 2. *C. freundii* and *K. pneumoniae* M5a1 were generously provided by T.H. Wilson and F. Ausubel, respectively. Cells harboring plasmids encoding the *C. freundii* or *K. pneumoniae* lac permease were also generously provided by T.H. Wilson. Plasmid encoding the *E. coli* raffinose operon was generously contributed by R. Schmitt. Plasmid encoding sucrose permease with the *E. coli* lac permease C-terminus as an epitope tag was constructed as described (Sahin-Tóth et al., 1995).

[1-^{14}C]Lactose, [α-^{35}S]dATP, and [^{125}I]protein A were from Amersham. [^{14}C(U)]Sucrose and [^{3}H]raffinose were purchased from DuPont NEN. [^{3}H]Melibiose was a generous gift from Gérard Leblanc. Deoxyoligonucleotides were synthesized on an Applied Biosystem 391 DNA synthesizer. All restriction endonucleases, T4 DNA ligase, Taq DNA polymerase were from New England Biolabs. DNA Sequenase was from United States Biochemical. Rabbit polyclonal antiserum against C-terminus of lac permease (Carrasco et al., 1984b) was prepared by Babco. mAb 4B1 was purified from ascites fluid by Protein A-Sepharose affinity chromatography (Sun et al., 1996). All other materials were reagent grade and obtained from commercial sources.

Mutant construction

Residues Asn245 to Arg259 (encoded by AAC TTT TTT ACA AGT TTC TTC GCA ACA GGC GAA CAA GGA ACG CGC) in loop VII/VIII of *E. coli* lac permease were used to replace to the homologous region (GTC TTT TAT GCA GGT TTA TTC GAA TCA CAC GAT GTA GGA ACG CGC) in *E. coli* sucrose permease. The mutant was constructed by site-specific mutagenesis of *cscB* in plasmid pSP72 (Sahin-Toth et al., 1995) by using a two-stage polymerase chain reaction (PCR; Ho et al., 1989). Mutants S249G, T253S, and G254P in *E. coli* lac permease were constructed in plasmid pT7-5 with cassette *lacY* encoding C-less permease (van Iwaarden et al., 1991) by one-stage PCR. Mutations were verified by sequencing the length of the PCR-generated segment using dideoxynucleotide termination and synthetic sequencing primers (Sanger et al., 1977) after alkali denaturation (Hattori & Sakaki, 1986).

Growth of bacteria

E. coli T184 (*lacZ*$^-$*Y*$^-$) transformed with plasmid encoding a given transporter were grown aerobically at 37 °C in Luria-Bertani broth with ampicillin (100 μg/mL) and streptomycin (10 μg/mL) except for *E. coli* T184 expressing the raffinose permease, which

Table 2. *Bacterial strains and plasmids*[a]

Strain	Genotype	Source
C. freundii	*lacY*$^+$	Okazaki et al., 1994
K. pneumoniae M5a1	*lacI*$^+$*lacZ*$^+$*lacY*$^+$	F. Ausabel and P. Yorgey
Plasmid		
pT7-5/*lacY*	*E. coli lacY*$^+$, *amp*^{r}	van Iwaarden et al., 1991
pSP72/*cscB*	*E. coli cscB*$^+$, *amp*^{r}	Sahin-Toth et al., 1995
pRU600/*rafB*	*E. coli rafR'A*$^+$*B*$^+$ *D*$^+$, *Cm*^{r}	Aslanidis and Schmitt, 1990
pNOT41/*lacY*	*C. freundii lacY*$^+$, *amp*^{r}	Lee et al., 1994
pRE510/*lacY*	*K. pneumoniae lacY*$^+$, *amp*^{r}	McMorrow et al., 1988

[a] *lacI*, lac repressor; *lacZ*, β-galactosidase; *lacY*, lac permease; *cscB*, chromosomally encoded sucrose permease; *rafR*, raffinose repressor; *rafA*, α-galactosidase; *rafB*, raffinose permease; *rafD*, sucrose hydrolase; *amp*, ampicillin; *Cm*, chloramphenicol.

was grown in the presence of chloramphenicol (34 μg/mL) and streptomycin (10 μg/mL). *C. freundii* and *K. pneumoniae* M5a1 were grown in Luria-Bertani broth without antibiotics. Overnight cultures were diluted 10-fold and allowed to grow for two hours before induction with 0.5 mM IPTG. After additional growth for two hours, cells were harvested by centrifugation.

Preparation of spheroplasts and 4B1 binding

Spheroplasts were prepared by lysozyme-EDTA treatment as described (Sun et al., 1996). Aliquots [0.5 mL containing 0.3 mg/mL protein in 100 mM potassium phosphate (KPi; pH 7.5)/0.5 M sucrose/5% bovine serum albumin (BSA)] were mixed with 5 μL of purified 4B1 (5 mg/mL), incubated at room temperature for one hour, centrifuged, washed once in incubation buffer without BSA, and resuspended in 0.4 mL of incubation buffer. Two microliters of [^{125}I] protein A (30 mCi/mg; 100 mCi/mL) were added, and incubation was continued for 45 min. The spheroplasts were then centrifuged, washed once by centrifugation, and resuspended to 50 μL in incubation buffer without BSA. Bound radioactivity was measured by liquid scintillation spectrometry using Scintsafe™ Econo 1 cocktail buffer.

Preparation of right-side-out (RSO) membrane vesicles

RSO membrane vesicles were prepared by osmotic lysis of spheroplasts prepared with lysozyme and EDTA (Kaback, 1971; Short et al., 1975).

Transport assays

Active transport in RSO membrane vesicles was assayed under oxygen in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (PMS) (Konings et al., 1971; Kaback, 1974). Membrane vesicles were suspended in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ buffer to a concentration of 2 mg/mL total protein, and [^{14}C] lactose (10 mCi/mmol), [^{3}H] melibiose (40 mCi/mmol) or [^{3}H] raffinose (50 mCi/mmol) were used at a final concentration of 0.4 mM as indicated.

Quantitation of lac and sucrose permease

Spheroplast samples containing *E. coli* lac or sucrose permease (with the lac permease C-terminus as an epitope; Sahin-Tóth et al., 1995) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Newman et al., 1981), and immunoblot analyses were carried out by using rabbit polyclonal antibody against the C-terminus of lac permease (Carrasco et al., 1984b). The amount of permease was quantitated with PhosphorImager Model 425F (Molecular Dynamics) as described (Sun et al., 1996).

Protein determination

Protein concentrations were determined as described (Peterson, 1977) with BSA as standard.

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References

Aslandis C, Schmitt, R. 1990. Regulatory elements of the raffinose operon: Nucleotide sequences of operator and repressor genes. *J Bacteriol 172*:2176–2180.

Bockmann J, Heuel H, Lengeler JW. 1992. Characterization of a chromosomally encoded, non-PTS metabolic pathway for sucrose utilization in *E. coli* EC3132. *Mol Gen Genet 235*:22–32.

Carrasco N, Viitanen P, Herzlinger D, Kaback HR. 1984a. Monoclonal antibodies against the *lac* carrier protein from *Escherichia coli*. 1. Functional studies. *Biochemistry 23*:3681–3687.

Carrasco N, Herzlinger D, Mitchell R, DeChiara S, Danho W, Gabriel TF, Kaback HR. 1984b. Intramolecular dislocation of the COOH terminus of the *lac* carrier protein in reconstituted proteoliposomes. *Proc Natl Acad Sci USA 81*:4672–4676.

Dunten RL, Sahin-Toth M, Kaback HR. 1993. Role of the charge pair formed by aspartic acid 237 and lysine 358 in the lactose permease of *Escherichia coli*. *Biochemistry 32*:3139–3145.

Frillingos S, Kaback HR. 1996a. Chemical rescue of Asp237→Ala and Lys358→Ala mutants in the lactose permease of *Escherichia coli*. *Biochemistry 35*:13363–13367.

Frillingos S, Kaback HR. 1996b. Monoclonal antibody 4B1 alters the pK_a of a carboxylic acid at position 325 (helix X) of the lactose permease of *Escherichia coli*. *Biochemistry 35*:10166–10171.

Frillingos S, Sahin-Tóth M, Lengeler JW, Kaback HR. 1995. Helix packing in the sucrose permease of *Escherichia coli*: Properties of engineered charge pairs between helices VII and XI. *Biochemistry 34*:9368–9373.

Frillingos S, Sahin-Toth M, Persson B, Kaback HR. 1994. Cysteine-scanning mutagenesis of putative helix VII in the lactose permease of *Escherichia coli*. *Biochemistry 33*:8074-8081.

Frillingos S, Wu J, Venkatesan P, Kaback HR. 1997. Binding of ligand or monoclonal antibody AB1 induces discrete changes in the lactose permease of *E. coli*. *Biochem*, in press.

Hattori M, Sakaki Y. 1986. Dideoxy sequencing method using denatured plasmid templates. *Anal Biochem 152*:1291–1297.

He MM, Voss J, Hubbell WL, Kaback HR. 1995a. Use of designed metal binding sites to study helix proximity in the lactose permease of *Escherichia coli*. 2. proximity of helix IX (Arg302) with helix X (His322 and Glu325). *Biochemistry 34*:15667–15670.

He MM, Voss J, Hubbell WL, Kaback HR. 1995b. Use of designed metal-binding sites to study helix proximity in the lactose permease of *Escherichia coli*: 1. Proximity of helix VII (Asp237 and Asp240) with helices X (Lys319) and XI (Lys358). *Biochemistry 34*:15661–15666.

Herzlinger D, Viitanen P, Carrasco N, Kaback HR. 1984. Monoclonal antibodies against the *lac* carrier protein from *Escherichia coli*. 2. Binding studies with membrane vesicles and proteoliposomes reconstituted with purified *lac* carrier protein. *Biochemistry 23*:3688–3693.

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene 77*:51–59.

Jung K, Jung H, Wu J, Privé GG, Kaback HR. 1993. Use of site-directed fluorescence labeling to study proximity relationships in the lactose permease of *Escherichia coli*. *Biochemistry 32*:12273–12278.

Jung K, Voss J, He M, Hubbell WL, Kaback HR. 1995. Engineering a metal binding site within a polytopic membrane protein, the lactose permease of *Escherichia coli*. *Biochemistry 34*:6272–6277.

Kaback HR. 1971. Bacterial membranes. *Methods Enzymol XXII*:99–120.

Kaback HR. 1974. Transport in isolated bacterial membrane vesicles. *Methods Enzymol 31*:698–709.

Kaback HR. 1983. The *lac* carrier protein in *Escherichia coli*: From membrane to molecule. *J Membr Biol 76*:95–112.

Kaback HR. 1989. Molecular biology of active transport: From membranes to molecules to mechanism. *Harvey Lect 83*:77–103.

Kaback HR. 1992. In and out and up and down with the lactose permease of *Escherichia coli*. In: Friedlander M, Mueckler M, eds. *International review of cytology*, Vol. 137A. New York: Academic Press. pp 97–125.

Kaback HR. 1996. The lactose permease of *Escherichia coli*: Past, present and future. In: Konings WN, Kaback HR, Lolkema JS, eds. *Handbook of biological physics II: Transport in prokaryotic and eukaryotic organisms*. Amsterdam: Elsevier. pp 203–227.

King SC, Hansen CL, Wilson TH. 1991. The interaction between aspartic acid 237 and lysine 358 in the lactose carrier of *Escherichia coli*. *Biochem Biophys Acta 1062*:177–186.

Konings WN, Barnes E Jr, Kaback HR. 1971. Mechanisms of active transport in isolated membrane vesicles. 2. The coupling of reduced phenazine methosulfate to the concentrative uptake of β-galactosides and amino acids. *J Biol Chem 246*:5857–5861.

Lee JL, Hwang PP, Hansen C, Wilson TH. 1992. Possible salt bridges between transmembrane α-helices of the lactose carrier of *Escherichia coli*. *J Biol Chem 267*:20758–20764.

Lee J, Okazaki N, Tsuchiya T, Wilson TH. 1994. Cloning and sequencing of the gene for the lactose carrier of *Citrobacter freundii*. *Biochem Biophys Res Commun 203*:1882–1888.

Lee JI, Varela MF, Wilson TH. 1996. Physiological evidence for an interaction between Glu-325 and His-322 in the lactose carrier of *Escherichia coli*. *Biochim Biophys Acta 1278*:111–118.

McMorrow I, Chin DT, Fiebig K, Pierce JL, Wilson DM, Reeve ECR, Wilson TH. 1988. The lactose carrier of *Klebsiella pneumoniae* M5a1: The physiology of transport and nucleotide sequence of the *lac Y* gene. *Biochim Biophys Acta 945*:315–323.

Newman MJ, Foster DL, Wilson TH, Kaback HR. 1981. Purification and reconstitution of functional lactose carrier from *Escherichia coli*. *J Biol Chem 256*:11804–11808.

Okazaki N, Tsuda M, Wilson TH, Tsuchiya T. 1994. Characterization of the lactose transport system in *Citrobacter freundii*. *Biol Pharm Bull 17*:794–797.

Peterson GL. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem 83*:346–356.

Poolman B, Konings WN. 1993. Secondary solute transport in bacteria. *Biochim Biophys Acta 1183*:5–39.

Sahin-Tóth M, Dunten RL, Gonzalez A, Kaback HR. 1992. Functional interactions between putative intramembrane charged residues in the lactose permease of *Escherichia coli*. *Proc Natl Acad Sci USA 89*:10547–10551.

Sahin-Tóth M, Frillingos S, Lengeler JW, Kaback HR. 1995. Active transport by the *cscB* gene product in *Escherichia coli* K-12. *Biochem Biophys Res Commun 208*:1116–1130.

Sahin-Tóth M, Kaback HR. 1993. Properties of interacting aspartic acid and lysine residues in the lactose permease of *Escherichia coli*. *Biochemistry 32*:10027–10035.

Sanger F, Nicklen S, Coulsen AR. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA 74*:5463–5467.

Short SA, Kaback HR, Kohn LD. 1975. Localization of D-lactate dehydrogenase in native and reconstituted *Escherichia coli* membrane vesicles. *J Biol Chem 250*:4291–4296.

Sun J, Li J, Carrasco N, Kaback HR. 1997. The last two cytoplasmic loops in the lactose permease of *Escherichia coli* comprise a discontinuous epitope for a monoclonal antibody. *Biochemistry 36*:274–280.

Sun J, Wu J, Carrasco N, Kaback HR. 1996. Identification of the epitope for monoclonal antibody 4B1 which uncouples lactose and proton translocation in the lactose permease of *Escherichia coli*. *Biochemistry 35*:990–998.

Teather RM, Bramhall J, Riede I, Wright JK, Furst M, Aichele G, Wilhelm V, et al. 1980. Lactose carrier protein of *Escherichia coli*. Structure and expression of plasmids carrying the Y-gene of the *lac* operon. *Eur J Biochem 108*:223–231.

van Iwaarden PR, Pastore JC, Konings WN, Kaback HR. 1991. Construction of a functional lactose permease devoid of cysteine residues. *Biochemistry 30*:9595–9600.

Wilmot CM, Thornton JM. 1988. Analysis and prediction of the different types of β-turn in proteins. *J Mol Biol 203*:221–232.

Wu J, Kaback HR. 1996. A general method for determining helix packing in membrane proteins *in situ*: Helices I and II are close to helix VII in the lactose permease of *Escherichia coli*. *Proc Natl Acad Sci USA 93*:14498–14502.

Wu J, Perrin D, Sigman D, Kaback H. 1995. Helix packing of lactose permease in *Escherichia coli* studied by site-directed chemical cleavage. *Proc Natl Acad Sci USA 92*:9186–9190.

Wu J, Voss J, Hubbell WL, Kaback HR. 1996. Site-directed spin labeling and chemical crosslinking demonstrate that helix V is close to helices VII and VIII in the lactose permease of *Escherichia coli*. *Proc Natl Acad Sci USA 93*:10123–10127.