Association between the Amino- and Carboxyl-Terminal Halves of Lactose Permease is Specific and Mediated by Multiple Transmembrane Domains

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Received October 19, 1995; Revised Manuscript Received December 8, 1995[⊗]

ABSTRACT: Lactose permease of *Escherichia coli* is a polytopic membrane transport protein containing 12 membrane-spanning segments. When the amino (N6)- and carboxy (C6)-terminal halves are expressed as separate gene fragments, association of the first half (N6) of the permease with the second half (C6) is necessary for stable insertion of C6 [Bibi, E., & Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 4325−4329]. In this report we demonstrate that N6−C6 interaction is specific, since N6 fragments derived from the structurally related tetracycline or sucrose transporters are unable to stabilize insertion of C6 from lactose permease. Furthermore, this association appears to be mediated by multiple transmembrane domains, since co-expression of progressively truncated N-terminal fragments (N5, N4, N3, N2, N1) with C6 leads to markedly decreased, but detectable amounts of C6 in the membrane. The results indicate that the N- and C-terminal six transmembrane domains of lactose permease are integrated into the membrane as separate units, and insertion of the C-terminal half is directed by specific interactions with the N-terminal half of the protein.

Accumulating evidence indicates that polytopic membrane proteins preserve their tertiary structure even when the peptide backbone is cleaved, indicating that forces between different domains within these proteins are able to maintain the three-dimensional organization in the membrane. In support of this concept is the observation that a number of proteins, including lactose permease (Bibi & Kaback, 1990; Wrubel et al., 1990; Zen et al., 1994), bacteriorhodopsin (Liao et al., 1984; Popot et al., 1987), the β-adrenergic receptor (Kobilka et al., 1988), a voltage-dependent Na^+ channel (Stühmer et al., 1989), the yeast α-factor transporter STE6 (Berkower et al., 1991), and adenylate cyclase (Tang et al., 1991), can be synthesized in two or more fragments that reconstitute to form a catalytically active complex. One of the better characterized models, the lactose (lac) permease of *Escherichia coli*, is a polytopic membrane transport protein with 12 hydrophobic transmembrane domains [see Kaback (1992)]. Bibi and Kaback (1990) showed that co-expression of lac permease fragments corresponding to the first (N6) and second (C6) halves of the molecule led to active transport while expression of either half independently did not. Similar observations were made when the permease was cleaved at different cytoplasmic and periplasmic loops, indicating that structural symmetry of the two halves is not a prerequisite for functional association (Wrubel et al., 1990; Zen et al., 1994). When the N6 and C6 fragments are expressed individually, N6 is observed inconsistently and C6 is not observed at all. Upon co-expression of N6 and C6, however, both fragments are found in the membrane. On the basis of these observations, it was suggested that N6 and C6 stabilize each other against proteolytic degradation. Though functional association of independently synthesized peptide fragments seems to be a general phenomenon, the observation that the N- and C-terminal halves of lac permease also stabilize each other upon association is unique thus far. It is unclear, however, whether the N6−C6 association is mediated by specific or nonspecific (e.g., hydrophobic) interactions between the two fragments. In this context, it has been proposed that interactions between α-helices of polytopic transmembrane proteins are mostly of a nonspecific, hydrophobic nature (Lemmon & Engelman, 1994). In the present study, we demonstrate that the N6−C6 association in lac permease is a result of the specific interaction of multiple transmembrane domains. We believe this eventually leads to stable membrane integration of the catalytically active complex.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose and [^{35}S]methionine were purchased from Amersham (Arlington Heights, IL). Oligonucleotides were synthesized on an Applied Biosystems 392 DNA synthesizer. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of lac permease (Carrasco et al., 1984b) was prepared by BabCo (Richmond, CA). All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. E. coli HB101 [*hsdS20 (r^-_B,m^-_B),recA13,ara-14,proA2,lacY1,galK2,rpsL20(Sm^r),xyl-5,mtl-1,supE44,* $^-$/F$^-$] (Boyer & Roulland-Dussoix, 1969) was used as carrier for the plasmids described. *E. coli* T184 [*$lacI^+O^+Z^-Y^-$(A), rpsL,met^-,thr^-,recA,hsdM,hsdR/F′,$lacI^q$-$O^+Z^{D118}(Y^+A^+)$*] (Teather et al., 1980) harboring plasmid pSP72/*lacY* with given alterations in the *lacY* gene was used for expression from the *lac* promoter by induction with isopropyl 1-thio-β-D-galactopyranoside (IPTG). The cassette *lacY* gene (EMBL-X56095) containing the *lac* promoter/operator was used for all *lacY* gene manipulations. For the

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⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1996.

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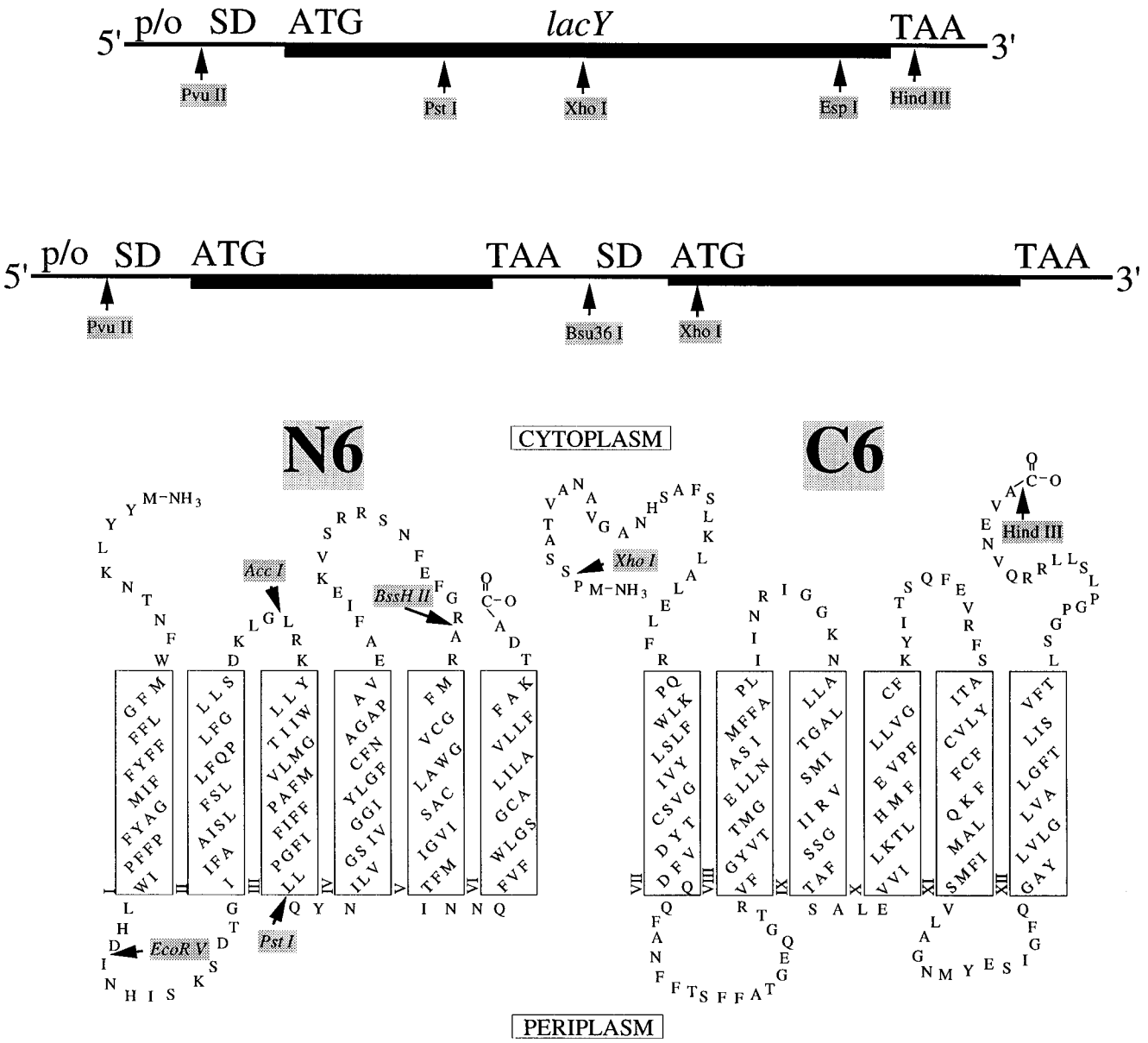

FIGURE 1: Gene organization and secondary structure model of N6−C6 "split" permease. The single-letter amino acid code is used, and putative transmembrane helices are shown in boxes. Restriction endonuclease sites used for construction of different split genes are indicated. p/o, promoter/operator; SD, Shine−Delgarno site; ATG, initiator methionine; TAA, termination codon.

constructs used in this paper, the cassette *lacY* gene has been modified so that the 5′ flanking *Bam*HI site has been removed and the 5′ wild-type non-translated sequence restored. This modification results in higher levels of expression [see Sahin-Tóth et al. (1995)]. The *lacY* gene with the *lac* promoter/operator region was cloned into plasmid pSP72 (Promega) between the *Eco*RI and *Hin*dIII sites. To preserve several unique restriction sites within the *lacY* gene, recognition sites for *Xho*I, *Pvu*II, and *Eco*RV have been eliminated in the polylinker region of pSP72.

Construction of Split Permease Genes. The split *lacY* gene encoding N6−C6 (see Figure 1) was constructed by inserting a short "splitting sequence" after codon 191 using PCR mutagenesis. A mutagenic antisense oligonucleotide complementary to codons 174−191 was synthesized. The mutagenic primer carried a nonhybridizing 5′ overhang containing a termination codon (TAA), a unique *Bsu*36I restriction site (CCTAAGG) overlapping with the 5′ upstream sequence of the *lacY* gene including a Shine−Delgarno site (TAAGGAAATCCATT), an initiator codon (ATG), and codons 192−196 of *lacY* (CCCTCGAGTGCCACG), including a *Xho*I restriction site. Using this antisense primer and a sense primer complementary to codons 50−56 upstream of the *Pst*I site, a 480 base pair (bp) fragment was amplified by PCR using Vent DNA polymerase. The PCR product was purified by Wizard PCR Purification System (Promega) and digested with *Pst*I and *Xho*I. The 300 bp digestion product was isolated and ligated into pSP72/*lacY* treated with the same enzymes.

Construction of N6 and C6 Gene Fragments. To construct the C6 gene, pSP72/*lacY* was digested with *Pvu*II and *Xho*I and self-ligated via a short linker containing the 5′ non-translated sequence of *lacY*, an initiator Met codon, and the codon for Pro192, the second amino acid residue of C6. The gene for N6 was constructed by digesting pSP72/*lacY*/N6C6 with *Bsu*36I and *Hin*dIII (see Figure 1), followed by treatment with Klenow fragment of DNA polymerase I and T4 DNA ligase. N6 with the lac permease C-terminal

epitope attached was engineered by digestion of pSP72/*lacY* with *Xho*I and *Esp*I, Klenow treatment, and ligation.

Split Genes Containing N-Terminal Fragments from tetB and cscB. Using plasmid pSP72/*lacY*/N6−C6, other split genes encoding N6 fragments from *tetB* (tetracycline transporter) or *cscB* (sucrose permease, Bockmann et al., 1992) genes of *E. coli* and C6 of *lacY* were engineered. Sense oligonucleotides complementary to codons 1−14 of the *tetB* or codons 1−11 of the *cscB* gene were synthesized with a 32 nucleotide long 5′ overhang corresponding to the *lacY* 5′ non-translated sequence including a unique *Pvu*II restriction site. Antisense primers, hybridizing to codons 177−186 of the *tetB* and codons 182−194 of the *cscB* gene, carried an overhang encoding a termination codon (TAA) and a unique *Bsu*36I restriction site. Gene fragments were amplified by PCR from plasmids pBR322 (*TetB*) and pT7−5/*cscB* (Sahin-Tóth et al., 1995). The PCR products were purified, digested with *Pvu*II and *Bsu*36I, and ligated into pSP72/*lacY*/N6−C6 treated with the same enzymes.

Split Genes with Truncated N-Terminal Fragments. To create the polynucleotide encoding N5, an antisense oligonucleotide complementary to codons 156−167 of *lacY* with a 5′ overhang containing a termination codon (TAA) and a *Bsu*36I restriction site was synthesized. Using a sense primer complementary to codons 50−56 upstream of the *Pst*I site, a 370 bp fragment was amplified by PCR using Vent DNA polymerase. The purified PCR product was digested with *Pst*I and *Bsu*36I, and the 220 bp product was ligated into pSP72/*lacY*/N6−C6 treated with the same enzymes. N4, N3, N2, and N1 gene fragments were constructed by synthesizing linkers encoding a termination codon (TAA), a unique *Bsu*36I restriction site (CCTAAGG) overlapping with the 5′ upstream sequence of the *lacY* gene including a Shine−Delgarno site (TAAGGAAATCCATT), and a start codon (ATG). Plasmid pSP72/*lacY* was digested with *Bss*HII and *Xho*I (N4), *Pst*I and *Xho*I (N3), *Acc*I and *Xho*I (N2), or *Eco*RV and *Xho*I (N1), and the restriction sites were ligated together using the appropriate linkers.

DNA Sequencing. Double-stranded plasmid DNA prepared by Wizard Minipreps (Promega) was sequenced after alkaline denaturation (Hattori & Sakaki, 1986) using the dideoxynucleotide termination method (Sanger et al., 1977; Sanger & Coulson, 1978) and synthetic sequencing primers. Each construct was verified by sequencing the length of the synthetic linker or the PCR-generated segment through the ligation junctions.

Active Transport. Active lactose transport was measured in *E. coli* T184 (Z^-Y^-) transformed with a given plasmid. Fully grown overnight cultures of cells were diluted 10-fold and grown aerobically for 2 h at 37 °C in the presence of 100 μg of ampicillin/mL. Expression of lac permease was then induced by addition of 0.5 mM IPTG (final concentration), and the cultures were grown for an additional 1 h. Cells were harvested by centrifugation, washed with 100 mM potassium phosphate (KP_i; pH 7.5)/10 mM $MgSO_4$, and assayed by rapid filtration (Consler et al., 1990).

Preparation of Membranes. E. coli T184 cells harboring given plasmids were resuspended in osmotic shock buffer [25 mM Tris-HCl (pH 8.0)/45% sucrose/1 mM ethylenediaminetetraacetate], kept on ice for 20 min, harvested by centrifugation, resuspended in cold distilled water, and kept 10 min on ice before adding 0.1 mg of lysozyme/mL. After incubation for 20 min, the suspensions were sonicated for 10 s. Unlysed cells were removed by low-speed centrifugation, and a crude membrane fraction was obtained after a 45 min ultracentrifugation at 350 000*g* in a Beckman Optima TL ultracentrifuge.

Immunological Analyses. Membrane fractions were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Newman et al., 1981). Proteins were transferred electrophoretically to a PVDF membrane (Millipore) and then probed with site-directed polyclonal antibody against the C terminus of lac permease (Carrasco et al., 1984b).

[^{35}S]Methionine Labeling. Labeling of lac permease with [^{35}S]methionine ([^{35}S]Me) and pulse−chase experiments were carried out *in vivo* using the T7 polymerase system as described by McKenna et al. (1991).

Protein Determinations. Protein was assayed in the presence of SDS by a modified Lowry procedure (Peterson, 1977).

RESULTS

Interaction of N6 with C6 Is Required for Membrane Integration of C6 and Active Lactose Transport. The *lacY* gene was cleaved into two halves in the middle cytoplasmic loop by inserting a short "splitting sequence" by PCR mutagenesis as detailed in Materials and Methods [see also Zen et al. (1994)]. The splitting sequence contains a termination codon, a new Shine−Delgarno site, and an initiator Met codon. This split *lacY* gene gives rise to a polycistronic message encoding the N-terminal (N6, residues 1−191) and the C-terminal (C6, residues 192−417) half of the protein as separate polypeptides (Figure 1). The split *lacY* gene was cloned under the *lac* promoter/operator and expression was induced by isopropyl 1-thio-β-D-galactopyranoside. As shown previously (Bibi & Kaback, 1990), co-expression of N6 and C6 leads to the formation of a catalytically active duplex, resulting in levels of lactose transport comparable to wild-type, intact permease (Figure 2A). Immunological analysis reveals that, when the two halves of the permease are expressed as individual polypeptides, N6 is associated with the membrane fraction in a stable form while C6 is not detected (Figure 2B). Importantly, C6 is never detected in cytoplasmic fractions of T184 cells (data not shown). Co-expression of both halves yields high membrane levels of C6.

Absence of C6 from Membranes Is Due to an Insertional Defect. There are at least two possible explanations for the absence of detectable C6 from T184 cells when expressed without N6: (1) C6 is inserted into the membrane normally and then rapidly degraded (association with the independently inserted N6 fragment prevents proteolysis); or (2) membrane targeting and/or insertion of C6 is defective, and the synthesized C6 is rapidly degraded in the cytoplasm (interaction with N6 in the cytoplasm or during insertion directs C6 into the membrane, thereby protecting it from degradation). To distinguish between the two possibilities, we examined the relative stability of C6 in the membrane by pulse−chase analysis. Specific metabolic labeling of C6 with [^{35}S]-methionine was carried out using the T7 promoter/polymerase system in the presence of rifampicin. As shown on Figure 3, even at the zero time point of the chase (i.e., before addition of unlabeled methionine) no C6 fragment is observed in membrane fractions of cells expressing C6 alone, indicating that C6 is not integrated. Under the conditions

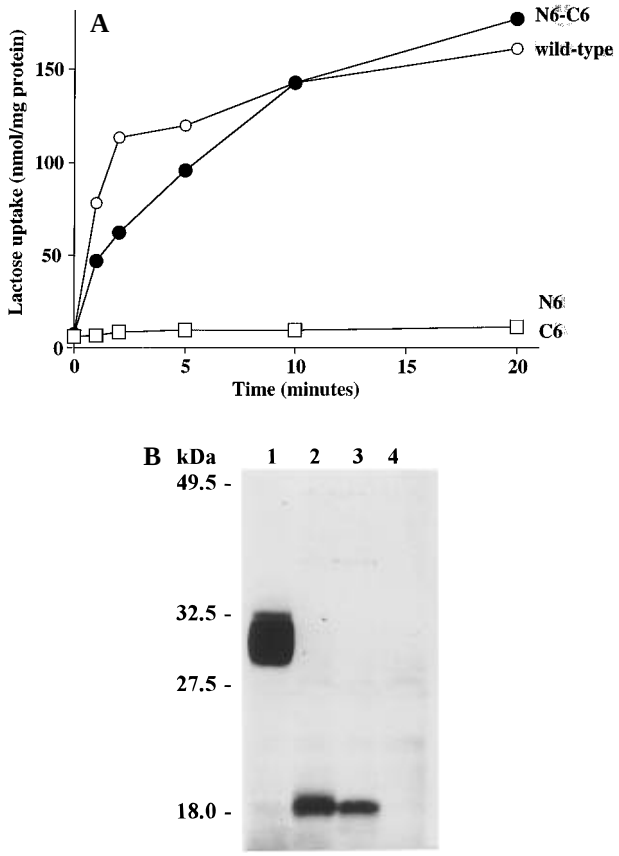

FIGURE 2: Transport activity and expression of wild-type, N6, C6, or N6−C6 permease. Cells were grown at 37 °C and induced with IPTG as described in Materials and Methods. (A) Lactose transport. Aliquots of cell suspensions (50 μL containing 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at room temperature. Transport was initiated by the addition of [1-^{14}C]lactose (10 mCi/mmol) to a final concentration of 0.4 mM. Reactions were quenched by addition of 3.0 mL of 100 mM KP_i (pH 5.5)/100 mM LiCl and rapidly filtered through Whatman GF/F filters. (B) Western blot of membrane fractions. Membranes were prepared as described in Materials and Methods, and samples containing approximately 100 μg of protein per sample were subjected to SDS polyacrylamide gel electrophoresis, electroblotted to PVDF membrane (Millipore), and incubated with anti-C-terminal antibody. After incubation with horseradish peroxidase-conjugated protein A and a short incubation with luminescent substrate (Amersham), the membrane was exposed to film for 1 min. Lane 1, wild-type lac permease; lane 2, N6−C6; lane 3, N6; lane 4, C6. In order to visualize the N6 gene product, an N6 construct was prepared that contained the C-terminal 12 amino acids of full-length permease (see Materials and Methods). Hence, N6 in lane 3 (but not in lane 2) contained the same epitope as C6 and both fragments are visualized on these blots with the same antiserum.

used no labeled C6 was detected in cytoplasmic fractions either (data not shown), suggesting that rapid proteolysis of the C6 fragment occurs. In contrast, high levels of C6 and N6 are detected in membrane fractions when the two fragments are co-expressed. The N6−C6 complex appears to be stable for at least 2 h after addition of unlabeled methionine.

Interaction between N6 and C6 Is Specific. To test whether or not the interaction between N6 and C6 is specific, C6 of lac permease was co-expressed with N6 fragments from two structurally related transport proteins. The tetracycline transporter (*tetB*) and a sucrose permease (*cscB*) from *E. coli* are both members of the same 12-transmembrane domain superfamily of transporters to which the lac permease belongs (Marger & Saier, 1993). When fragments containing

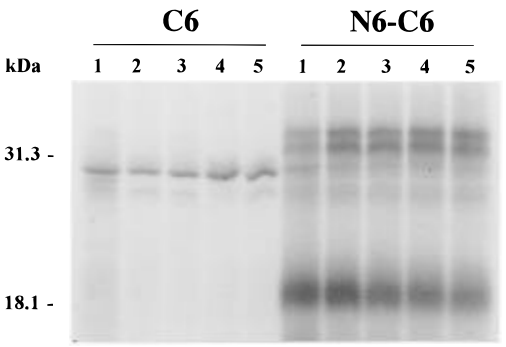

FIGURE 3: Stability of C6 and N6−C6 permeases assessed by [^{35}S]-methionine pulse−chase analysis. *E. coli* T184 cells were co-transformed with pGP1-2 encoding T7 RNA polymerase under the control of a heat-shock promoter and pSP72/*lacY* encoding C6 or N6−C6. Cells were grown at 30 °C and heat-shocked for 60 min at 42 °C. After incubation with [^{35}S]methionine at 30 °C for 3 min, an aliquot was removed as the zero time point (lane 1). A 200-fold excess of cold methionine was then added, and aliquots were removed for membrane preparation at 1 min (lane 2), 5 min (lane 3), 30 min (lane 4), and 2 h (lane 5). Membrane fractions (approximately 50 μg of membrane protein) were subjected to polyacrylamide gel electrophoresis, and the dried gel was exposed to film for 12 h.

the first six transmembrane domains of these proteins are co-expressed with C6 from lactose permease, no C6 is detected in the membrane (data not shown), indicating that the N6 fragments from proteins other than lac permease are unable to direct C6 of lac permease to the membrane. These observations suggest that the functional association between N6 and C6 is mediated by specific interactions.

Multiple Transmembrane Domains in N6 Interact with C6. In an attempt to identify the region of N6 primarily responsible for the specific interaction with C6, N6 was progressively truncated, and the N-terminal fragments (N5, N4, N3, N2, N1) were co-expressed with C6. As predicted, none of the shorter fragments form a functional duplex with C6, as indicated by the lack of transport activity when N5, N4, N3, N2, or N1 is co-expressed with C6 (not shown). In contrast, although to a markedly decreased extent, each N-terminal fragment is capable of promoting the membrane integration of C6 (Figure 4). Removal of one transmembrane domain from N6 (N5) causes a significant decrease in the amount of C6 detected in the membrane. Shortening of N5 to N4 further diminishes the amount of C6 inserted. Compared to N4, additional truncations (N3, N2, or N1) gradually further decrease membrane levels of C6, although changes are less pronounced. Interestingly, co-expression of as little as the first transmembrane domain (N1) with C6 is enough to promote low but significant membrane insertion of C6.

DISCUSSION

Previous observations and data presented here strongly suggest that interaction of N6 with C6 is required for stable insertion of the active split permease duplex (Bibi & Kaback, 1990; Zen et al., 1994). However, the nature of the interaction has not been studied in detail. Are there specific regions on C6 and N6 that mediate their association, or, alternatively, is the association due to nonspecific hydrophobic helix pairing? Under physiological conditions, random association of hydrophobic domains is unlikely, however, nonphysiologically high concentrations of hydro-

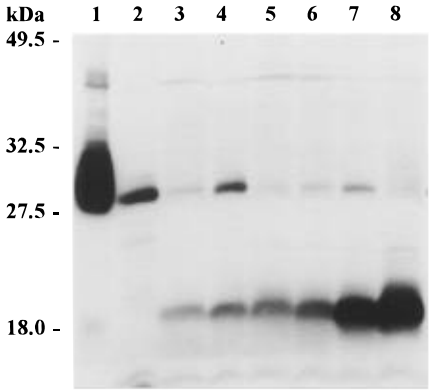

FIGURE 4: Effect of different N-terminal fragments on the membrane insertion of C6. Progressively truncated forms of N6 (N5, N4, N3, N2, and N1) have been co-expressed with C6, and the amount of C6 inserted into the membrane quantitated on immunoblots. Cell growth, induction, membrane preparation, electrophoresis, and Western blotting was carried out as described in Materials and Methods and Figure 2. Lane 1, wild-type lac permease; lane 2, C6; lane 3, N1−C6; lane 4, N2−C6; lane 5, N3−C6; lane 6, N4−C6; lane 7, N5−C6; lane 8, N6−C6. The sharp band around 30 kDa is due to nonspecific reaction of the antiserum.

phobic proteins may favor this type of interaction. In this respect, it is noteworthy that overexpression appears to be a prerequisite for efficient N6−C6 association and transport activity (Bibi & Kaback, 1990). Therefore, it seems possible that overexpressed hydrophobic segments from other proteins might interact with C6 and facilitate membrane insertion. To test this hypothesis, C6 of lac permease was co-expressed with N6 fragments from the tetracycline transporter (*tetB*) and a sucrose permease (*cscB*) from *E. coli*. These proteins are members of the same superfamily of transporters to which the lac permease belongs (Marger & Saier, 1993). The heterologous N6 fragments were unable to direct C6 of lac permease to the membrane, indicating that the functional association between N6 and C6 requires specific interactions. Subsequently, in an effort to delineate which parts of N6 are important for N6−C6 association, progressively truncated forms of N6 were co-expressed with C6. Surprisingly, all of the truncated N-terminal fragments were able to stabilize C6 to some extent. The amount of C6 inserted seems to decrease in a gradual fashion between N6 and N1 (see Figure 4). Quantitation of the individual bands by phosphorimaging (data not shown) indicates that the most marked changes occur between N4 nd N6. On the basis of this observation, and assuming comparable levels of expression for the different N-terminal fragments [see Stochaj et al. (1988)], we speculate that at least three transmembrane domains (1, 5, and 6) play an important role in the interaction with C6. Since both the first and the sixth transmembrane domains seem to be part of N6 that interacts with C6, the N6−C6 interaction must occur before N6 is fully integrated into the membrane. Similarly, multiple domains of C6 must participate in the interaction with N6, indicating that both N6 and C6 form independent "insertional cassettes". In this respect, on the basis of the observation that a salt bridge between transmembrane domains VII and XI (Asp237−Lys358) is important for membrane integration of the permease, it has been previously suggested that the last six transmembrane helices are inserted as a unit (Dunten et al., 1993; Sahin-Toth & Kaback, 1993).

It is intriguing to speculate that membrane insertion of the N6−C6 complex may occur by the same mechanism as intact wild-type permease. Interestingly, wild-type permease seems to be "naturally split", i.e., N6 and C6 are connected by a relatively large hydrophilic cytoplasmic loop which may be expanded in size by insertion of foreign sequences or whole genes (McKenna et al., 1991; Consler et al., 1993; Privé et al., 1994) without affecting membrane insertion or function of the protein. In addition, a number of eukaryotic polytopic membrane proteins [e.g., P-glycoprotein, CFTR, sodium channel, yeast STE6 transporter; see Friedlander and Mueckler (1993)] are characterized by groups of six hydrophobic transmembrane domains (TM) separated by large hydrophilic stretches. Recent data obtained for the P-glycoprotein (A. Borel and S. Simon, submitted for publication) and opsin (S. Simon and M. Friedlander, manuscript in preparation) suggest that nascent polypeptide chains are translocated across the endoplasmic reticulum membrane sequentially, but are not actually integrated into the phospholipid bilayer until synthesis of 6−7 TMs is completed. Protein synthesis in the ribosome occurs in an aqueous environment and by permitting chain extension and translocation to occur in a similar, water-filled membrane channel, molecular interactions associated with proper folding are facilitated under more energetically favorable conditions. A number of recent studies support the presence of a water-filled translocation channel and that the translocating peptide chain is not in direct contact with lipid (Gilmore & Blobel, 1985; Simon & Blobel, 1991). The concept of partitioning into lipid as a late event in protein translocation is relatively new, but it could explain the dependency of the permease C-terminal half on the N terminus for membrane integration. Alternatively, the N-terminal half may specifically interact with chaperone-like molecules that may serve to prevent aggregation or misfolding prior to synthesis of the entire insertional "cassette"; the C-terminal half alone may not be able to facilitate this interaction. Taken as a whole, the observations on the properties of the N6−C6 split permease provide indications that membrane insertion of the C-terminal half of lac permease is directed by specific interactions with the N-terminal half of the protein.

ACKNOWLEDGMENT

We are grateful to Christine Kincaid for technical support and assistance in the preparation of this manuscript. We also thank Gunter Blobel, Sheila Fallon, and Sandy Simon for helpful discussions.

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BI952496G