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The Hydrophobic Domain of Cytochrome b_5 Is Capable of Anchoring β-Galactosidase in *Escherichia coli* Membranes

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Cytochrome b_5 is inserted posttranslationally into membranes in vivo and spontaneously into liposomes in vitro by a short carboxyl-terminal hydrophobic membrane-anchoring sequence. DNA corresponding to this hydrophobic sequence has been synthesized, and two gene fusions with the *Escherichia coli* enzyme β-galactosidase have been constructed by locating the hydrophobic domain in one case at the *Eco*RI site near the C terminus and in the other at the normal C terminus of the enzyme. The latter fusion protein was enzymatically active, having approximately 50% of the specific activity of β-galactosidase, and cells expressing this protein grew normally with lactose as the sole carbon source. Both fusion proteins were localized to the *E. coli* inner membrane, converting β-galactosidase from a cytoplasmic enzyme to a membrane-associated enzyme. The hydrophobic domain of cytochrome b_5 therefore contains the information required to target polypeptides containing this domain to the membrane. Use of the cytochrome b_5 hydrophobic peptide, either alone or in conjunction with other localizing sequences such as signal sequences, provides a general procedure for associating proteins with membranes. Polypeptides bearing this hydrophobic peptide may have considerable use as pharmaceuticals when associated with liposomes or cellular membranes.

Proteins can be redirected to different subcellular locations or exported from cells by addition or replacement of DNA that contains various forms of targeting signals. One such targeting signal is the N-terminal signal peptide, which in eucaryotic cells serves to direct the protein across the endoplasmic reticulum and in bacterial cells, across the inner membrane to be localized in either the periplasmic space or the outer membrane. Other targeting signals permit proteins to be transported into mitochondria, chloroplasts, and peroxisomes. By joining DNA coding for a targeting signal to DNA coding for an arbitrary protein, the protein will in general be targeted to a new location determined by the targeting signal (for a review, see reference 20).

Cytochrome b_5 is an amphipathic liver microsomal membrane protein that may have a general membrane-targeting signal because it apparently inserts into any membrane available to it. It lacks a signal sequence and is synthesized on cytoplasmic ribosomes, indicating that cytochrome b_5 inserts into membranes posttranslationally (1). The polypeptide consists of two distinct domains linked by a trypsin-sensitive segment. The amino-proximal domain is the catalytic domain, involved in electron transport, and the hydrophobic carboxyl-proximal domain (HP) serves to anchor the protein in the membrane (17).

Cytochrome b_5 is known to insert spontaneously into preformed artificial membrane bilayers and liver microsomal membranes (13, 17), but this property is lost if the hydrophobic domain is proteolytically altered or removed (5). When expressed in bacteria, rat liver cytochrome b_5 was found in the ultracentrifuged membrane pellet fraction, suggesting that it may be membrane associated (21). These observations indicate that the short hydrophobic domain possesses the property of spontaneous membrane association and suggest that any protein attached to the cytochrome b_5 hydrophobic domain should be targeted to membranes.

We report here that in *Escherichia coli*, a hybrid enzyme consisting of β-galactosidase linked at its normal C terminus to the hydrophobic region of rabbit liver cytochrome b_5 is found associated with the inner membrane. This finding indicates that an arbitrary protein or enzyme could be associated with the inner aspect of the inner membrane by linkage to the cytochrome b_5 hydrophobic domain. We suggest that by combining a signal sequence at the N terminus with the hydrophobic domain at the C terminus, many proteins, although not β-galactosidase (12), could become associated with the outer aspect of the inner membrane or with the outer membrane. In bacteria with a single membrane, a similarly engineered enzyme could presumably be located external to the cell so that a permeability barrier would not limit access to its substrate. Such modified bacteria might have potential use as immobilized enzyme systems, having as one advantage continual renewal of the enzyme by protein synthesis.

In eucaryotic cells, addition of a glycosylphosphatidyl inositol membrane anchor to a secreted protein results in targeting to the plasma membrane, with the protein oriented into the extracellular space (3). Such a procedure probably could not be easily accomplished in bacteria, since no procaryotic proteins anchored by glycosylphosphatidyl inositol are known. Moreover, if the addition of the glycosylphosphatidyl inositol anchor takes place in the lumen of the rough endoplasmic reticulum, it would not be possible to target such proteins to the inner aspect of the eucaryotic plasma membrane, whereas a protein bearing the cytochrome b_5 hydrophobic domain could potentially localize at this site.

(A preliminary communication based partly on this work has appeared elsewhere [S. K. George, R. P. Sandoval,

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TABLE 1. *E. coli* K-12 strains

Strain	Characteristics	Source or reference
MB392	(F′ *lacI*^{q}ZΔM15 *proAB traD36*) *hsdR514* (r_K^- m_K^+) *supE44 supF58* Δ(*argF-lac*)*U169 galK2 galT22 metB1 trpR55 pro*::Tn*5* Kan^r	M. Benedik, Biology Department, Texas A&M University
MC4100	F$^-$ *araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	16
CQ24Δ3	F$^-$ *lacI*^{q} *lacZ*Δ3 *araD139 leu gal* His *argG* pURE *rpsL150 xyl met trp mtl-1 ilv thi*	As for MB392

L. A. Benson, and G. M. Ihler, Proc. 1988 Miami BioTechnology Winter Symp., ICSU Short Rep. **8**:33, 1988].)

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* K-12 strains (Table 1) were grown at 37°C in L broth containing ampicillin or kanamycin (50 μg/ml each) or in M9 supplemented with L-amino acids (50 μg/ml) and lactose (0.2% [wt/vol]). Isopropylthiogalactoside (IPTG) was used at a final concentration of 1 mM.

HP DNA. A DNA sequence that would code for the hydrophobic domain of rabbit liver cytochrome b_5 (Fig. 1A; 9) was selected by using codons found frequently in highly expressed *E. coli* genes (6). Four oligonucleotides were synthesized (Biology Department, Texas A&M University) with sufficient overlap to permit efficient hybridization (Fig. 1B). After deprotection, polyacrylamide gel purification, and phosphorylation, the oligonucleotides were annealed and primer extended with Klenow polymerase to yield duplex DNA (Fig. 1C). Single-strand breaks were repaired with T4 DNA ligase, and the DNA was cleaved with *Bam*HI and *Sal*I at restriction sites incorporated in the sequence. This DNA was cloned in reverse orientation into the multiple cloning site in pUC19 (19) to yield pUC19:HP (Fig. 2A). In this orientation, the cloned DNA is not transcribed after transformation into *E. coli* MB392, and a choice of restriction sites is available for subcloning.

The *Bam*HI-*Sal*I fragment was excised from pUC19:HP and cloned between compatible sites in bacteriophage M13mp18 and M13mp19 replicative-form DNA. After generation of single-stranded M13 template DNA in each case, dideoxy sequencing (15) confirmed that the cloned DNA corresponded to the HP gene sequence. However, a single clone (HPm1) was isolated that had two substitutions in its sequence, a C to T at nucleotide 32 and a G to A at nucleotide 85. This feature alters amino acid residues 11 (Thr to Ile) and 29 (Ala to Thr) of the sequence shown in Fig. 1A and corresponds to conserved residues in cytochrome b_5 sequences from several species (10).

Construction of *lacZ*-HP fusion genes. Fusion genes were constructed so that the hydrophobic domain of cytochrome b_5 would be located at the carboxyl terminus of the protein, which reproduces the location in cytochrome b_5 and permits the utilization of the existing promoter and ribosome-binding site. The sequence of the normal termination codon for cytochrome b_5 is not known. We chose a UAG codon as a terminator so that the HP DNA could also be used at the amino terminus of a fusion gene, with extension of the polypeptide beyond the UAG codon being permitted by propagation in bacteria containing amber suppressors. A *Hin*dIII-*Bam*HI fragment carrying part of the *trp* operon and the *lac* operon genes *Z*, *Y*, and *A* was excised from pMC81 (Table 2) and cloned into the *Hin*dIII and *Bam*HI sites downstream of *lacPO* in pSKG1 (a derivative of pBH20), which had the *Eco*RI site deleted by cleavage, primer extension, and blunt-end ligation to yield pSKG2 (Table 2). The HP sequence on a *Bam*HI-*Sal*I fragment was excised from pUC19:HP and purified by polyacrylamide gel electrophoresis. After primer extension with Klenow polymerase

A. Amino acid sequence of the hydrophobic domain (HP).

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1         10        20        30        40
LSKPMETLITTVNSNSSWWTNWVIPAISALIVALMYRLYMADD.
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B. Four Chemically Synthesized Molecules.

1. 5'GCGGCGGCGGATCCACTGTCCAAACCGATGGAAACCCTGATCACCACCGTTAACTCCAACTCC3'
2. 5'CGGGATAACCCAGTTGGTCCACCAAGAGGAGTTGGAGTTAACGGT3'
3. 5'TCTTGGTGGACCAACTGGGTTATCCCGGCTATCTCCGCTCTGATCGTTGCTCTGATGTACCGT3'
4. 5'ATATATAGTCGACTCCTAATCGTCAGCCATGTACAGACGGTACATCAGAGCAACGATCAGAGCGGAGATAGC3'

C. Duplex DNA Prepared by Hydridization and Ligation of Nicks

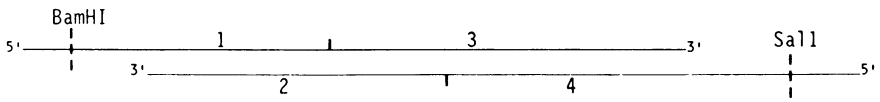

FIG. 1. (A) Amino acid sequence corresponding to that of HP DNA, based on the sequence reported for the membranous segment of rabbit liver microsomal cytochrome b_5 (9). (B) Constituent synthetic oligonucleotides hybridized to yield duplex DNA (C). Incorporated into the sequence, proximal to the *Sal*I site, is a TAG stop codon at the C terminus of the cytochrome b_5 sequence.

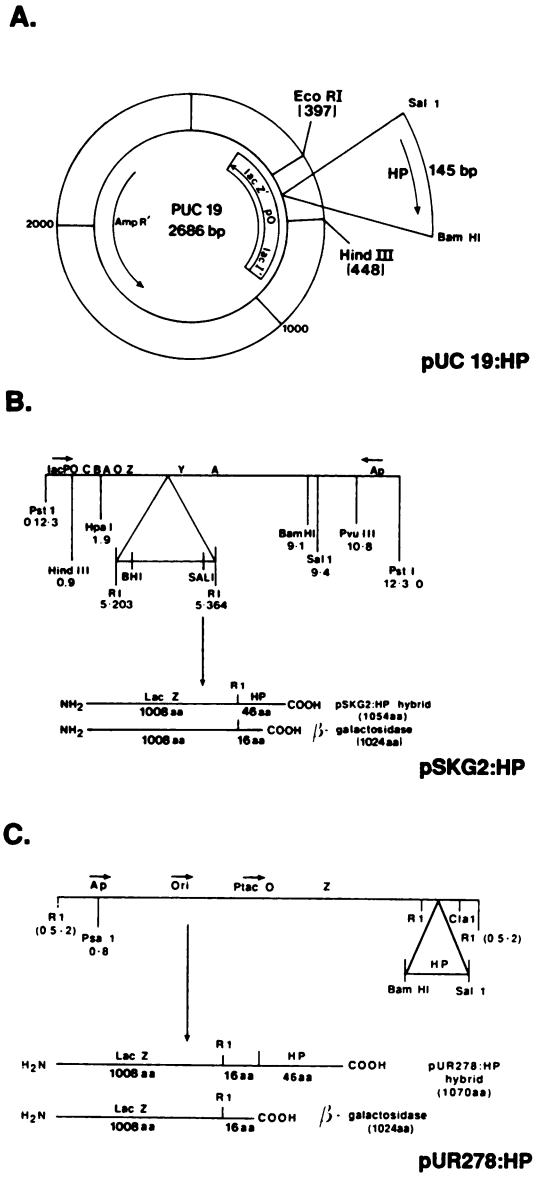

FIG. 2. Plasmid constructs. HP DNA was excised from pUC19:HP (A) and cloned into the C-terminal *Eco*RI site of *lacZ* to give pSKG2:HP (B). The size of the expected fusion protein (1,054 amino acids [aa]) compared with that of β-galactosidase is indicated. Cloning of the HP DNA at the normal C terminus of *lacZ* yields pUR278:HP (C), and the resulting fusion protein is expected to consist of 1,070 amino acids.

and blunt-end *Eco*RI linker addition, the DNA was inserted into the unique *Eco*RI site located near the C terminus of *lacZ* in pSKG2 to create pSKG2:HP (Fig. 2B), which codes for a fusion protein (1,054 amino acids) bearing 1,008 β-galactosidase amino acids (lacking the C-terminal 16 amino acids) and 46 new C-terminal amino acids, of which 43 code for the hydrophobic peptide of cytochrome b_5.

Gene fusions created by using the pUR cloning vectors developed by Ruther and Muller-Hill (14) were also constructed. pUR278 (Table 2) contains a multiple cloning site, with unique cloning sites (*Bam*HI, *Sal*I, *Xba*I, and *Hin*dIII) located immediately after the AAA codon corresponding to the C-terminal *lacZ* amino acid, lysine. The unique *Bam*HI and *Sal*I sites permit excision from pUC19:HP and in-frame

TABLE 2. Plasmids used in constructing gene fusions

Plasmid	Characteristics	Source or reference
pUC19:HP	HP inserted between *Bam*HI and *Sal*I sites in pUC19; 2,820 base pairs	This study
pMC81	Ap^r *trp*(*CBA*) lacZYA; 25,600 base pairs	4
pBH20	Ap^r Tc^r *lacPO*; 4,570 base pairs	8
pSKG2	pBH20 deleted of its *Eco*RI site and carrying the *trp*CBA *lac*ZYA segment from pMC81 but lacking a 346-base-pair segment containing part of Tc; 12,220 base pairs	This study
pSKG2:HP	HP inserted at the *Eco*RI site of *lacZ* in pSKG2; 12,381 base pairs	This study
pUR278	Ap^r *lacPOZ*; 5,200 base pairs	14
pUR278:HP	HP inserted at the C-terminal *Bam*HI and *Sal*I sites of *lacZ* in pUR278; 5,342 base pairs	This study

cloning of the HP sequence, locating HP at the normal C terminus of β-galactosidase. The resulting plasmid, pUR278:HP, codes for a fusion protein (1,070 amino acids) bearing the complete LACZ sequence (1,024 amino acids) and extended at the C terminus by 46 amino acid residues, the latter 43 corresponding to HP (Fig. 2C). Both plasmids pSKG2:HP and pUR278:HP were transformed into *E. coli* MB392, MC4100, and CQ24Δ3. Maxiprep DNA corresponding to each of the plasmids were isolated, and the sequence across the junction of each of the fusions was determined by using oligonucleotide 2 (a constituent of the HP DNA) as a primer (Biology Department sequencing facility, Texas A&M University), which verified the fusions to be in frame.

Location of LACZ-HP fusion proteins. Cells transformed with the respective plasmids were grown in 25 ml of L broth to an optical density at 600 nm of 0.3 and induced with IPTG at a final concentration of 1 mM, after which growth was allowed to continue to an optical density of 1.0. The cells were collected by centrifugation and suspended in 1 ml of ice-cold 0.01 M Tris acetate buffer (pH 8.1). Phenylmethylsulfonyl fluoride was added to 1 mM, and the cells were immediately passed twice through a French pressure cell at 16,000 lb/in^2. Unlysed cells and debris were removed by low-speed centrifugation (2,000 × *g* for 5 min), and the lysate was separated into supernatant and membrane fractions either by ultracentrifugation (180,000 × *g* for 1 h at 4°C in a TLA 100.2 rotor) or by flotation through sucrose. In the latter case, the lysate was made 70% (wt/vol) with respect to sucrose (0.5 ml), loaded beneath a sucrose step gradient consisting of 2.5 ml of 65% (wt/vol) sucrose and 0.5 ml of 40% (wt/vol) sucrose and ultracentrifuged for 4 h at 244,000 × *g* (45,000 rpm) in an SW50.1 rotor at 15°C. The gradients were fractionated from the bottom into 0.5-ml portions. The cytoplasmic and membrane fractions were analyzed by determination of β-galactosidase enzymatic activity and also by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) (18), using a monoclonal antibody to β-galactosidase (Promega Biotec, Milwaukee, Wis.). Several detergents {Triton X-100, SDS, 3[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate, octylglucoside, sodium cholate, sodium deoxycholate, Nonidet P-40, and Tween 80} were assessed for the ability to solubilize the membrane-associated protein.

TABLE 3. Distribution of β-galactosidase activity

Source	% of total β-galactosidase activity (mean ± SEM)	
	Membrane	Supernatant
IPTG-induced MB392 cells carrying: (n = 6)		
pUR278	20.3 ± 4.3	79.7 ± 4.3
pUR278:HP	90.6 ± 1.9	9.4 ± 1.9
pUR278:HPml	89.3 ± 0.7	10.7 ± 0.7
Isolated CQ24Δ3 cell membranes (n = 4)[a]		
LACZ (pUR278)	6.7 ± 0.06	93.3 ± 0.06
LACZ-HP (pUR278:HP)	95.6 ± 0.31	4.4 ± 0.31

[a] Membranes were incubated with normal β-galactosidase (pUR278) or the pUR278:HP-derived LACZ-HP fusion protein.

β-Galactosidase activity of LACZ-HP fusion proteins. The distribution of β-galactosidase activity between the membrane and supernatant fractions was assayed by the colorimetric *O*-nitrophenyl-β-D-galactopyranoside (ONPG) method (11). The specific activity of the fusion protein derived from pUR278:HP was also determined after purification by gel filtration on Sepharose 4B, followed by ion-exchange chromatography on DEAE-cellulose. The ability of the LACZ-HP fusion proteins to permit growth with lactose as the sole carbon source was assessed by measuring growth rates.

Immunocytochemical analysis. Induced cells were fixed in 25 ml of 0.5% glutaraldehyde (30 min at 4°C), washed in 0.01 M Tris acetate buffer (pH 8.1), and collected by centrifugation into molten soft agar (25°C, 2,000 × *g* for 10 min). Cells were embedded in LR-white, and ultrathin sections were cut and mounted on Formvar-coated nickel grids. After blocking of nonspecific antibody binding with a 2% bovine serum albumin solution (30 min at 25°C), the grids were incubated with primary antibody (polyclonal rabbit anti-β-galactosidase; Organon Teknika, Malvern, Pa.) for 2 h at 25°C. The grids were washed with buffer and incubated with secondary antibody (goat anti-rabbit polyclonal coupled to 10-nm gold beads; BioClin, Cardiff, United Kingdom) for 1 h at 25°C. The grids were then examined with a Philips 420 electron microscope.

Incubation with isolated bacterial membranes. The bacterial membranes from 200 ml of an IPTG-induced culture of CQ24Δ3 were isolated as described above for location of the LACZ-HP fusion protein. The membranes were suspended in 8 ml of 0.01 M Tris acetate buffer (pH 8.1), collected by ultracentrifugation (180,000 × *g* for 1 h at 4°C in a TLA 100.2 rotor), and resuspended in 8 ml of the same buffer. Approximately 50,000 U of either native β-galactosidase (pUR278) or LACZ-HP fusion protein (pUR278:HP) was incubated with 1 ml of the membrane suspension for 1 h at 37°C. Incubations were also carried out with the membranes present in French press lysates derived from CQ24Δ3 cells. Membranes were separated from the supernatant by ultracentrifugation or by flotation through sucrose, and fractions were analyzed for β-galactosidase enzymatic activity.

RESULTS

After lysis and ultracentrifugation to separate membrane and supernatant fractions, approximately 90% of the β-galactosidase enzymatic activity from bacteria containing the pUR278:HP or the pUR278:HPml fusion was associated with the membranes, whereas about 80% of normal β-galactosidase activity from bacteria containing pUR278 was found in the supernatant (Table 3). When the membrane and supernatant fractions were separated by flotation through sucrose, approximately 55% of the pUR278:HP-derived β-galactosidase activity copurified with membranes, and more than 90% of normal β-galactosidase activity was found in the supernatant fraction (Fig. 3). SDS-polyacrylamide gel electrophoresis demonstrated that the LACZ-HP fusion proteins were primarily associated with the membrane, in contrast to native β-galactosidase, which was mainly cytosolic (Fig. 4). The membrane-associated fusion proteins

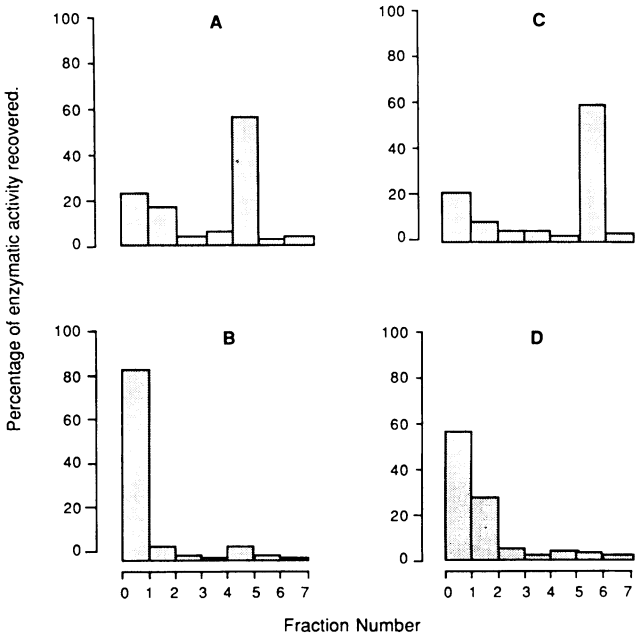

FIG. 3. Separation of membranes from supernatants by flotation through sucrose. Fractions represent 0.5-ml portions of the step gradient fractionated from the bottom and assayed for β-galactosidase activity. (A) MB392 cells containing pUR278:HP (n = 3); (B) MB392 cells containing pUR278 (n = 3); (C) CQ24Δ3 French press lysate incubated with detergent-extracted fusion protein from MB392 containing pUR278:HP (n = 4); (D) detergent-extracted fusion protein from MB392 containing pUR278:HP (n = 4).

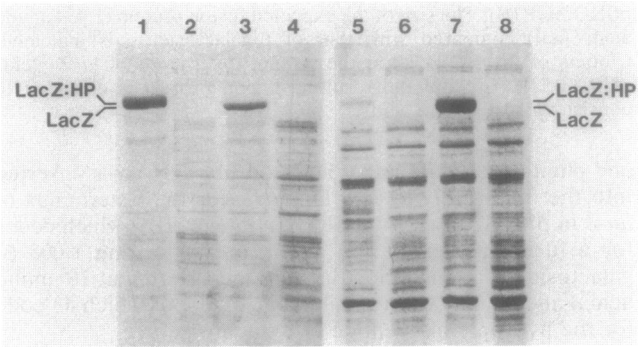

FIG. 4. SDS-polyacrylamide gel electrophoresis of membrane (lanes 1 to 4) and supernatant (lanes 5 to 8) fractions of cells expressing native β-galactosidase (pUR278) and the LACZ-HP fusion protein (pUR278:HP). Lanes 4 and 8, Uninduced cells containing pUR278; lanes 3 and 7, IPTG-induced cells containing pUR278; 2 and 6, uninduced cells containing pUR278:HP; lanes 1 and 5, induced cells containing pUR278:HP.

were readily solubilized with SDS, but other detergents proved less effective. The most effective nondenaturing detergent was sodium deoxycholate. Two consecutive extractions with 0.5-ml volumes of 2% sodium deoxycholate, accompanied by brief bursts of sonication in ice, solubilized most of the protein, with the residual debris being removed by centrifugation (65,000 × *g* for 1 h at 4°C in a TLA 100.2 rotor).

Cytochrome b_5 has been shown to insert into isolated liver microsomal membranes (17). Likewise, the pUR278:HP fusion protein, in contrast to β-galactosidase, spontaneously associated with washed *E. coli* membranes (Table 3 and Fig. 3). As will be demonstrated elsewhere, the fusion proteins also spontaneously associate with liposomes, so this result was not unexpected. However, this observation leaves open to question whether the fusion proteins are associated with the membranes in vivo or whether the association occurs only after cellular lysis.

Immunocytochemical studies on the cellular distribution of the fusion proteins and β-galactosidase demonstrated that the fusion proteins were associated with the membrane in vivo. The cellular location of fusion protein synthesized from pUR278:HP was revealed by electron microscopic localization of gold-labeled secondary antibody bound to the immune complex consisting of the primary antibody and the β-galactosidase immunoreactivity. The fusion protein was found to be localized almost exclusively to the periphery of the cell (Fig. 5C), and in some examples it was clear that the gold beads were associated with the inner membrane. In contrast, β-galactosidase was found uniformly distributed over the cytoplasm (Fig. 5B). Despite its altered location, the fusion protein was physiologically active, since MB392 cells containing either pUR278:HP or pUR278 had similar division times and growth properties in minimal medium containing lactose as the sole carbon source.

When whole-cell lysates from induced MB392 or CQ24Δ3 cells containing pUR278:HP were analyzed by Western blotting with anti-β-galactosidase antibody, using native β-galactosidase (116 kilodaltons [kDa]) (16) (Fig. 6, lanes 3 and 7) and the ω fragment of β-galactosidase (112 kDa) (Fig. 6, lanes 1, 3, and 4) as molecular size markers, a polypeptide consistent in size with the expected LACZ-HP fusion protein (121 kDa) was seen (Fig. 6, lanes 8 and 9). The molecular sizes of the LACZ-HP polypeptide in both the membrane and supernatant fractions were identical (Fig. 4, lanes 1 and 5), indicating that proteolytic removal of the hydrophobic peptide had not occurred to any significant extent in the cytosol. When similar fractions from MC4100 and CQ24Δ3 containing pSKG2:HP were analyzed, a polypeptide of a size consistent with that expected (120 kDa) for this construct was seen (Fig. 6, lanes 5 and 6). MB392 containing pSKG2:HP yielded the same polypeptide (120 kDa) as the major species of fusion protein. The small amount of a higher-molecular-weight species seen only in strain MB392 may have resulted from suppression by *supE44* (present in MB392 only) at the UAG codon located distal to the cytochrome b_5 sequence. The next predicted terminator occurs in *lacY*, and the estimated molecular weight for this species corresponds to that observed for this polypeptide.

From a Coomassie blue-stained SDS-polyacrylamide gel calibrated with bovine serum albumin standards, the amount of fusion protein produced by pUR278:HP was estimated to be about 60% of the amount of β-galactosidase made by pUR278. The specific activity of the membrane-bound fusion protein was about 2.7×10^5 U/mg of fusion protein, using bovine serum albumin as a calibrated standard to estimate the quantity of fusion protein present, and that of β-galactosidase was 6.7×10^5 when determined similarly on the cytoplasmic fraction. The specific activity of the purified fusion protein was 2.5×10^5; the reported value for native β-galactosidase is 4.5×10^5 (16). These numbers indicate that addition of the hydrophobic peptide decreases modestly both the total protein synthesized and its specific activity.

DISCUSSION

DNA that would code for a polypeptide with the amino acid sequence of the cytochrome b_5 hydrophobic region was chemically synthesized and linked to DNA coding for β-galactosidase. Two constructs were prepared, one containing the entire nucleotide sequence of β-galactosidase (pUR278:HP) and a second containing only the first 3,024 nucleotides (pSKG2:HP). Because the carboxyl-proximal amino acids of β-galactosidase are required for enzymatic activity, the second construct does not yield an active enzyme. Since the pUR278:HP construct is enzymatically active, a charged carboxyl group at the normal C terminus of β-galactosidase is not required for activity, and the presence of additional amino acid residues beyond the normal carboxyl terminus indicates that the presence of a new domain does not appreciably interfere with appropriate folding and tetramerization.

The LACZ-HP fusion protein corresponding to the pUR278:HP construct is enzymatically active in vitro when assayed with ONPG as the substrate, although the measured specific activity is lower (50%) than that of normal β-galactosidase. Colonies containing this construct are blue on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal); when grown in medium containing lactose as the sole carbon source, bacteria expressing the hybrid protein are capable of growing at rates equal to those of isogenic bacteria containing normal β-galactosidase. The latter observation indicates that the fusion protein is physiologically active in vivo and can fully substitute for the normal enzyme.

The LACZ-HP fusion proteins corresponding to both constructs are localized in the bacterial membrane, from which they could not be released except by vigorous detergent solubilization. This contrasts with native β-galactosidase, which is a cytosolic enzyme. Membrane localization of a hybrid protein encoded by a *lacZ-pbpB* fusion has also been reported (2). After lysis of the cells and ultracentrifugation to collect the membranes, about 90% of the enzymatic activity derived from pUR278:HP is located in the membrane fraction, whereas only about 20% of the β-galactosidase activity from control bacteria sediments with the membranes. Membrane association was confirmed by demonstrating that the majority of the pUR278:HP-derived β-galactosidase activity, in contrast to normal β-galactosidase, copurified with membranes floated up in a sucrose density gradient. The fusion proteins, however, can spontaneously associate with bacterial membranes; therefore, this observation does not directly demonstrate that the proteins are membrane associated in vivo.

Immunocytochemical analysis of thin-sectioned bacteria clearly showed that protein encoded by pUR278:HP was localized almost exclusively in the membrane, whereas native β-galactosidase was uniformly distributed across the cytoplasm. Good evidence to suggest insertion of the fusion protein into bacterial membranes in vivo was thus obtained. In some thin sections, the inner membrane could be clearly differentiated from the outer membrane, and in those the fusion protein was clearly located in the inner membrane.

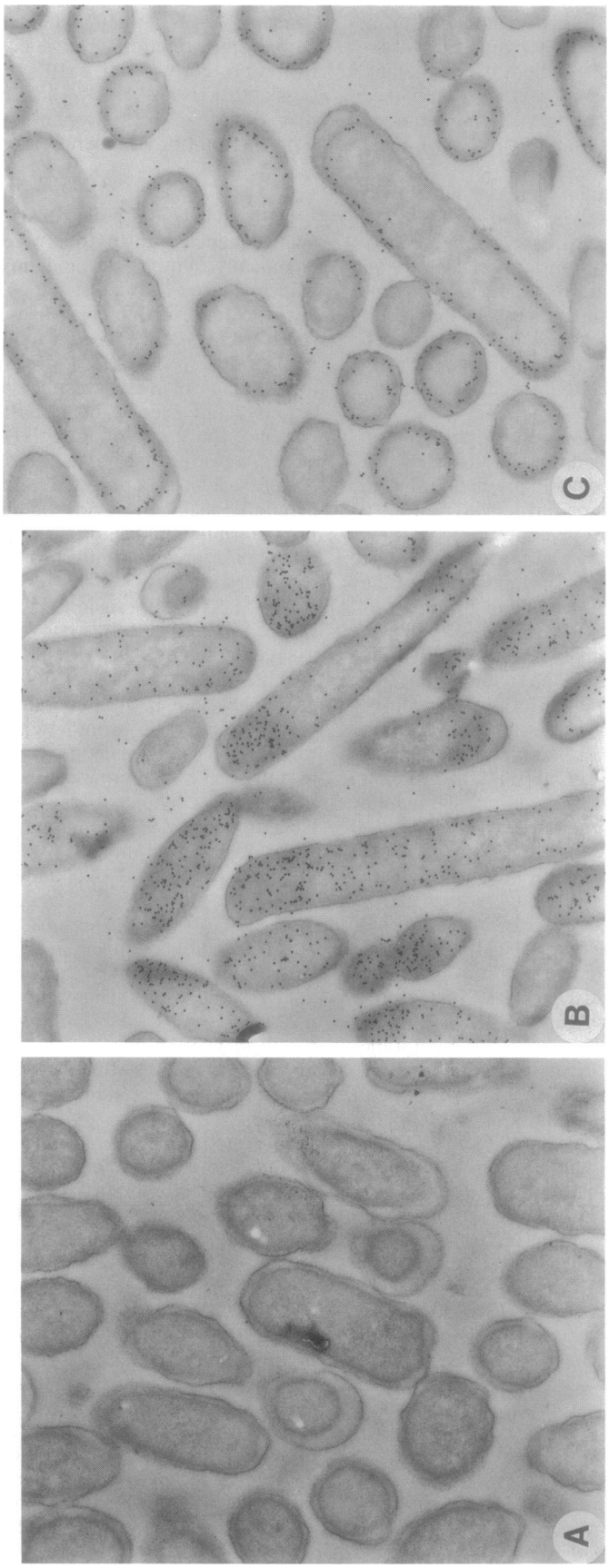

FIG. 5. Immunocytochemical localization of β-galactosidase immunoreactivity. (A) Control MB392 cells; (B) MB392 cells expressing native β-galactosidase (pUR278); (C) MB392 cells expressing the LACZ-HP fusion protein (pUR278:HP).

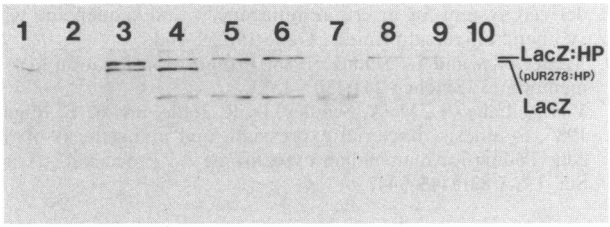

FIG. 6. Western blot of IPTG-induced whole cells demonstrating relative molecular weights of the expressed β-galactosidase immunoreactivities. Lanes: 1, MB392; 2, CQ24Δ3; 3, MB392(pSKG2); 4, MB392(pSKG2:HP); 5, MC4100(pSKG2:HP); 6, CQ24Δ3(pSKG2:HP); 7, MB392(pUR278); 8, MB392(pUR278:HP); 9, CQ24Δ3(pUR278:HP); 10, β-galactosidase marker. Analysis of corresponding membrane and supernatant fractions (data not shown) indicated that in contrast to both native β-galactosidase and its ω fragment, the majority of the expressed LACZ-HP fusion proteins were membrane associated.

One clone of synthesized DNA, HPml, proved to have two nucleotide substitutions (C to T and G to A), which resulted in the conservative amino acid changes Thr to Ile and Ala to Thr at residues 11 and 29 of the HP sequence shown in Fig. 1A. Although these amino acid residues are highly conserved among the cytochrome b_5 sequences known for various species, these substitutions appeared not to markedly affect the association of the fusion protein with the membrane, as judged by enzymatic activity and Western blot analysis. Experiments are in progress to determine the effect of changes of amino acid sequence, introduced synthetically or mutationally in HP, on the relative ability of the hydrophobic domain to direct membrane insertion in *E. coli*; a mutation decreasing the extent of membrane association has been isolated. Cytochrome b_5 from rat liver differs from that from rabbit at six amino acid residues (1, 2, 5, 13, 31, and 42) in the hydrophobic domain, with substitutions of Ile for Leu, Ala for Ser, Ser for Met, Glu for Asn, Val for Ile, and Glu for Asp, respectively. These changes are generally conservative, although the substitution of Glu for Asn introduces a negative charge at residue 13. However, this sequence is found in cytochrome b_5 from several other species (10), which suggests that residue 13 is not likely to be part of the region directly interacting with the membrane.

We anticipate that polypeptides bearing the cytochrome b_5 hydrophobic segment will be useful for in vivo studies involving cell and membrane physiology. In particular, since membrane insertion probably occurs posttranslationally, combining the cytochrome b_5 hydrophobic segment with an N-terminal signal sequence could lead to either transmembrane trapping of the protein or transmembrane transport of the entire polypeptide. In the latter case, the polypeptide might be anchored by its hydrophobic segment to the outer aspect of the inner membrane or to the outer membrane.

In work to be published elsewhere, we show that both cytochrome b_5 and the β-galactosidase–HP fusion protein associate spontaneously with preformed liposomes and also with the external face of membranes of erythrocytes and other cells; cytochrome b_5 appears to be inserted in the membrane, particularly since in collaborative studies with Reiner Peters (Max-Planck Institut, Frankfurt, Federal Republic of Germany) it was found to have the lateral diffusion properties of a highly mobile membrane protein. One possible application of this technology would be to extend the circulatory life span of infused proteins by insertion into erythrocytes, which have a 120-day life span (7). Antibodies for viruses and other infectious organisms could be inserted into erythrocyte membranes, which may provide long-term protection, and polypeptide receptors for hormones or other substances could be inserted in membranes of other cell types.

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