The fusion-related hydrophobic domain of Sendai F protein can be moved through the cytoplasmic membrane of *Escherichia coli*

(stop transfer/bacterial membrane protein/paramyxovirus/membrane fusion/hydrophobicity)

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ABSTRACT Recent work on a prokaryotic membrane protein, gene III protein (pIII) of coliphage f1, showed that polypeptide segments of sufficient hydrophobicity functioned to stop transfer of the polypeptide across the cell membrane: strings of 16 or more hydrophobic amino acids sufficed. A fusion-related hydrophobic domain (FRHD) of Sendai F protein, a sequence of 26 consecutive uncharged residues, has been implicated in the fusion of the viral membrane envelope and the target-cell membrane through a hydrophobic interaction. As it is located on the exterior of the viral membrane, this sequence must be transferred across the host-cell membrane during synthesis. We have inserted either the FRHD or the F protein membrane anchor (the COOH-terminal region of the F protein) into an internal site of a secreted pIII, which lacks its natural membrane anchor. These two hydrophobic sequences behave in the bacteria just as they do in their natural eukaryotic cell host. The F protein membrane anchor functions to stop transfer, conferring a membrane-spanning topology to the F-pIII hybrid protein; however, the FRHD is moved through the cytoplasmic membrane and derivatives carrying this sequence are secreted to the periplasm. We discuss how the FRHD is compatible with passage through the membrane and yet is still able to mediate membrane fusion through a presumed hydrophobic interaction.

Membrane proteins show great variety in how their polypeptide chains are arranged with respect to the lipid bilayer. Some simple transmembrane proteins span the bilayer one time, having their amino-terminal domains outside (transferred across the membrane) and their carboxyl-terminal domains left behind in the cytoplasm. Such proteins are synthesized with two hydrophobic segments: an amino-terminal signal peptide and a downstream sequence consisting of 20 or more uncharged residues—the membrane-spanning domain. The signal peptide initiates transfer of the polypeptide through the membrane (1). The ultimate positioning of the protein chain across the membrane depends on the termination of this transfer process. Such a “stop-transfer” event is specified by sequences that contain the hydrophobic membrane-spanning segment (2–4). Recently, Davis and Model (5) have demonstrated for a prokaryotic membrane protein, the gene III protein (pIII) of the coliphage f1 (M13 or fd), that it is simply the hydrophobicity of this domain and not some more subtle feature of structure or sequence that suffices to stop transfer. They suggested that any segment of the polypeptide that is sufficiently hydrophobic will act to stop transfer and serve to anchor the protein to the membrane.

Some eukaryotic simple transmembrane proteins have, in addition to a signal peptide and the downstream membrane anchor, a third hydrophobic domain—the fusion-related

hydrophobic domain (FRHD)—interposed between these two. These proteins, components of the envelopes of paramyxo- and myxoviruses, mediate fusion of viral and cell membrane, resulting in viral penetration and also virus-induced cell fusion and hemolysis (6). Synthesized as fusion-inactive precursors, these proteins are activated by a host cell protease prior to viral budding from the host plasma membrane (7–13); this activating cleavage is just to the NH₂-terminal side of the FRHD (14–16). The FRHD of Sendai fusion protein (F protein), a hydrophobic stretch of 26 uncharged residues, is highly conserved among paramyxoviruses (14, 17)—i.e., Sendai (18–20), simian virus 5 (21), Newcastle disease virus (17), mumps (22), measles (23), and parainfluenza 3 (P. L. Collins, personal communication). Several lines of evidence suggest that the FRHD mediates the fusion reaction by hydrophobic interaction with the lipid bilayer of the target membrane (17, 24–26). This domain, which is available on the virus exterior, must be moved through a cellular membrane of the host into the lumen of the rough endoplasmic reticulum during its synthesis. It is of interest how the FRHD sequence is transferred through the lipid bilayer during the membrane integration of these proteins. Recent work has shown that much shorter strings of 12–16 hydrophobic residues can function to anchor other proteins to the membrane (5, 27, 28). We have tested the hydrophobic domains of Sendai F protein for stop-transfer activity in a well-characterized prokaryotic system.

Gene III protein (pIII), a minor constituent of the bacteriophage f1 coat, is synthesized as an integral component of the *Escherichia coli* cytoplasmic (inner) membrane. It has an amino-terminal signal peptide of 18 residues, and a 23-residue-long hydrophobic membrane anchor near the carboxyl terminus. Removal of the anchor domain allows secretion of pIII to the periplasm—i.e., cell exterior. Stop-transfer function could be restored by inserting into the secreted protein strings of arbitrary hydrophobic amino acids; 16 or more uncharged residues will anchor the protein so that sequences downstream remain in the cytoplasm (5). In this report, we have inserted into a secreted version of pIII the hydrophobic domains from the Sendai F protein, the FRHD or the F protein membrane anchor. We find that these hydrophobic sequences behave in the bacterial milieu just as in their natural eukaryotic host; whereas the F protein membrane anchor acts to stop transfer, derivatives carrying the hydrophobic FRHD clearly are secreted across the membrane into the bacterial periplasm.

MATERIALS AND METHODS

***E. coli* Strains.** JM101 is $\Delta lacpro$, *supE*, *thi*/F'*traD36*, *proAB*, *lacI*^q Δ M15 (29). TB1 is a Sr1::Tn10 *recA* derivative of JM101 (from Angus Lamond).

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Abbreviations: FRHD, fusion-related hydrophobic domain; bp, base pair(s).

Oligonucleotides. Three *Bam*HI linkers, an 8-mer (CG-GATCCG), a 10-mer (CCGGATCCGG), and a 12-mer (CCCGGATCCGGG) (Collaborative Research, Waltham, MA) were used for plasmid construction. Two sequencing primers, complementary to either fl (+)-strand nucleotides 2245–2260, (GGTCAGACGATTGGCC), or (–)-strand nucleotides 2187–2202, (CAGAGACTGCGCTTTC), within gene III (30) were synthesized by the phosphoramidite method on an Applied Biosystems (Foster City, CA) 380A DNA synthesizer. Full-length oligonucleotides were purified by gel filtration through Sephadex G-75 in 100 mM triethylamine bicarbonate (pH 8.0).

Plasmid Construction. pND320 (27) inducibly expresses the amino-terminal 342 amino acids of pIII from the *lac uv5* promoter. Plasmids S4 and A21 (18) contain Sendai cDNA sequences encoding amino acids 99–330 and 443–565 of the F protein, respectively. DNA was digested, purified, and ligated by standard methods (31). For each of the three plasmids, whose construction is described below, *Bam*HI linkers were added to the ends of the F DNA fragments and inserted into the unique *Bam*HI site of pND320 (see Fig. 1), which had been dephosphorylated by treatment with bacterial alkaline phosphatase.

pDH301 contains the 78-base-pair (bp) *Hinf*I/*Mnl* I fragment encoding the FRHD of the F protein residues 117–142. This fragment was obtained as follows: (i) the ends of the 119-bp *Hinf*I fragment of S4 were filled with the Klenow fragment of DNA polymerase I, (ii) 12-nucleotide *Bam*HI linkers were added, (iii) digested with *Mnl* I, (iv) 8-nucleotide *Bam*HI linkers were added, and (v) linkers were activated by digestion with *Bam*HI.

pDH221 contains the 216-bp *Hpa* II/*Bst*NI fragments of S4, encoding residues 111–182 of the F protein. The sticky ends of this fragment were filled in with the Klenow fragment of DNA polymerase I, and 12-nucleotide *Bam*HI linkers were added before ligation to pND320.

pDH409 contains the 120-bp *Rsa* I/*Mnl* I fragment of A21 encoding F protein residues 496–535. This fragment was obtained as follows: (i) 10-nucleotide *Bam*HI linkers were added to the 174-bp *Rsa* I fragment of A21, (ii) digested with *Mnl* I, (iii) 8-nucleotide *Bam*HI linkers were added, and (iv) linkers were activated by *Bam*HI digestion. DNA sequencing showed that an extra 9 nucleotides (CGGAACCGG), presumably a defective linker, was inserted at the fusion joint of the F sequence and the 10-nucleotide linker; this translates to Pro-Glu-Pro and does not shift the downstream reading frame.

Ligation products of the three plasmid constructions were used to transform JM101. Ampicillin-resistant colonies were screened for F gene sequence by colony hybridization with the corresponding DNA fragment labeled with ³²P by nick-translation. Size and orientation of the insert at the *Bam*HI sites of each plasmid were analyzed by restriction digestion.

DNA Sequencing. The exact fusion joints and the sequence across the inserted F segment were determined by dideoxy sequencing (32). Single-stranded DNA templates of both polarities were derived from each plasmid DNA by *Exo* III digestion initiated from unique sites within the plasmid as described (27). For one polarity, *Exo* III digestion was initiated from the unique *Eco*RI site, and the fl (+)-strand primer was used. For the other strand, *Exo* III digestion was initiated from the unique *Hind*III site and the (–)-strand primer was used.

Protein Labeling, Alkali Fractionation, and Pronase Digestion. Bacteria transformed with each plasmid were grown to mid-logarithmic phase in minimal medium, induced for 5 min with 1 mM isopropyl β-D-thiogalactoside and pulse-labeled for 45 sec with [³⁵S]methionine (20–50 μCi per 0.2-ml culture; 1 Ci = 37 GBq) as described (5). For alkali fractionation, the pulse was terminated with 1 ml of ice-cold 0.1 M NaOH.

Samples were mixed vigorously with a Vortex and centrifuged in an Eppendorf Microfuge for 15 min to separate the pellet and the supernatant (33).

For Pronase digestion, 1 ml of pulse-labeled cells was spun for 30 sec in an Eppendorf Microfuge and immediately resuspended in 0.1 ml of ice-cold TS (20% sucrose/100 mM Tris-HCl, pH 8.0/10 mM EDTA) with 100 μg of lysozyme per ml, and incubated on ice for 10 min. Spheroplasts were aliquoted and incubated on ice for 1 hr with specified concentrations of Pronase (Calbiochem–Behring) freshly dissolved in TS. In some cases, Triton X-100 was added with the Pronase to a final concentration of 0.1%. To assess the integrity of the cell membrane, all samples were immunoprecipitated with antiserum to a cytoplasmic protein, thioredoxin. In the absence of detergent, >90% of the thioredoxin remained undigested.

Protein sample preparation, immunoprecipitation, and polyacrylamide gel electrophoresis were performed as described (5). ¹⁴C protein size standards were as follows: phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 26 kDa; lactoglobulin, 18 kDa (Bethesda Research Laboratories).

RESULTS

Plasmids Expressing F-pIII Hybrid Proteins. The plasmid pND320 encodes a truncated pIII, missing its carboxyl-terminal 82 amino acids. This protein has a functional signal sequence at its amino terminus but lacks its natural membrane anchor. When expressed in *E. coli*, it is secreted across the cytoplasmic membrane to the periplasmic space (5, 27). From pND320, we have constructed three plasmids—pDH221, pDH301, and pDH409—in which segments of the cloned F gene sequence (18) were inserted into the gene III coding sequences at the *Bam*HI site (Fig. 1). This site divides the gene III sequences in pND320 into upstream and downstream regions, encoding 213 and 129 amino acids, respec-

FIG. 1. Construction of genes that encode F-pIII hybrid proteins. The sequences of gene III [wild type (wt) and pND320-encoded] and Sendai F are shown as open boxes with their hydrophobic sequences indicated: the signal peptide of pIII and F (hatched box), the membrane anchor of pIII and F (stippled box), and the FRHD of the F protein (solid box). The plasmids pDH301, pDH409, and pDH221 were constructed by inserting the indicated F protein sequences into the *Bam*HI site of the truncated gene III coding sequences of pND320. The scale at the top indicates number of amino acids. Two segments of Sendai F protein are shown below. To the left are 38 residues including the uncharged FRHD (underlined). At right are the 32 residues including the hydrophobic F protein membrane anchor (underlined). Charged residues surrounding the hydrophobic sequences are indicated. Amino acids are designated by the single-letter code.

tively. *Bam*HI linkers 8, 10, or 12 nucleotides long were ligated to the ends of the inserted F sequences so as to preserve the correct reading frame through the hybrid genes. The structure of the three plasmids was confirmed by DNA sequencing.

The F sequence of pDH301 encodes just the 26 hydrophobic residues of the FRHD. The F segment of pDH221 encodes the FRHD plus the 6 amino acids to the amino-terminal side and the 40 amino acids to the carboxyl-terminal side of this sequence; this construct was designed to test the effect of the relatively large number of surrounding charged residues on the membrane topology of the FRHD (Fig. 1). The F-pIII hybrid protein encoded by pDH409 has the F protein membrane anchor sequence.

Alkali Fractionation of the Hybrid Proteins. Alkali fractionation (33) separates *E. coli* proteins into two classes (34). Most integral membrane proteins sediment with the pellet, as they maintain association with the membranes through the NaOH treatment. Periplasmic and cytoplasmic proteins, as well as proteins peripherally associated with membranes, fractionate to the supernatant. We have used this technique to assess the location of the F-pIII hybrid proteins in the cell (Fig. 2). Wild-type pIII, expressed by plasmid pND1 (27), fractionates with the alkali pellet, reflecting its integral association with the cytoplasmic membrane (Fig. 2; alkali treatment causes pIII to migrate as a doublet: N.G.D. and P. Model, unpublished results). In contrast, the pDH301- and the pDH221-encoded proteins, both of which contain the 26 hydrophobic amino acids of FRHD, fractionate to the supernatant like the parental pND320-encoded protein, consistent with secretion to the periplasm. The pDH409-encoded protein, with the F protein membrane anchor, fractionates to the alkali pellet as an integral membrane protein. In this case, the donated hydrophobic F sequence apparently can substitute for the deleted pIII membrane-spanning domain and anchor the hybrid protein to the membrane.

Membrane Topology of the Hybrid Proteins. It has been suggested that protein translocation, initiated from an amino-terminal signal sequence, proceeds linearly with the polypeptide transferred across the membrane from amino to carboxyl terminus (35). Sequences within the polypeptide that stop this process should leave the downstream portion of the polypeptide in the cytoplasm. We have used accessibility to Pronase digestion to probe the membrane topology of the F-pIII hybrid proteins. Parts of the polypeptides that have been transferred to the periplasm are susceptible to proteolysis; cytoplasmic domains are protected by the membrane.

Fig. 2. Cellular location of F-pIII hybrid proteins assayed by alkali fractionation. JM101 bacteria carrying one of five plasmids were pulse-labeled for 45 sec with [35 S]methionine. Lanes: 1, pND320; 2, pDH221; 3, pDH301; 4, pDH409; 5, pND1. Alkali treatment separated each into pellet (P) and supernatant (S) fractions. Samples were immunoprecipitated with antiserum to pIII and were electrophoresed on a NaDodSO₄/polyacrylamide gel.

As a control for this experiment, we have used a secreted and a membrane-bound mutant pIII, encoded by pND32 and pND35, respectively (5). Both plasmids have repeats of an oligonucleotide that translates to the hydrophobic tetrapeptide Leu-Ala-Leu-Val, inserted into the *Bam*HI site of pND320. The pND32-encoded protein has two repeats and the protein encoded by pND35 has five repeats. Four or more repeats, 16 or more hydrophobic residues, can function to anchor the truncated pIII to the membrane (5). The pND35-encoded protein gives rise to a protected cytoplasmic fragment when intact spheroplasts are treated with increasing concentrations of Pronase (Fig. 3A). The pND32-encoded protein does not.

The pDH221-encoded protein, which contains the FRHD plus the surrounding F sequence and which was fractionated by alkali to the supernatant, yields no Pronase-protected fragment (Fig. 3A). In terms of Pronase digestibility, it resembles quite closely the secreted periplasmic protein encoded by pND32. Some faint bands are present in all samples, whether or not they were treated with protease. Such contaminants are seen when high concentrations of the pIII antiserum are used. The pDH301-encoded protein, which contains just the FRHD, also gives no Pronase-protected fragment (data not shown). Clearly, the hydropho-

Fig. 3. Membrane topology of F-pIII hybrid polypeptides in intact cells assayed by Pronase accessibility. JM101 cells carrying one of three plasmids (pDH221, pDH409, or pND32) were pulse-labeled for 1 min with [35 S]methionine, treated with lysozyme to produce spheroplasts, and then treated with specified concentrations of Pronase in the presence or absence of Triton X-100. In a separate identical experiment, TB1 cells carrying pND35 were used. (A *recA*⁻ background is necessary to preserve the five tandem oligonucleotide repeats that encode the hydrophobic membrane anchor of the pND35-encoded protein.) In each case, digested samples were immunoprecipitated with the pIII antiserum and electrophoresed on a 6 M urea/NaDodSO₄/20% polyacrylamide gel. (A) Treatment with different concentrations of Pronase. Cells were treated with no Pronase (lanes a), 10 µg of Pronase per ml (lanes b), 100 µg of Pronase per ml (lanes c), or 1 mg of Pronase per ml (lanes d) as described. (B) Treatment with Pronase in the presence of detergent. Cells were treated with no Pronase (lanes a), 100 µg of Pronase per ml (lanes c), or 100 µg of Pronase per ml/1% Triton X-100 (lanes e). Positions of molecular size markers are indicated on the left in kDa. The aberrant NaDodSO₄/polyacrylamide gel mobility of pIII is well-documented (36–38). This anomaly is shared by various truncated pIII derivatives (2, 27), including the Pronase-protected carboxyl-terminal fragment of the pND35-encoded protein (5).

bic sequence of the FRHD can be transferred through the *E. coli* cytoplasmic membrane. The pDH409-encoded protein, which fractionates to the alkali pellet (Fig. 2), gives rise to a Pronase-protected fragment. This fragment is digested when the non-ionic detergent, Triton X-100, is added (Fig. 3B), indicating that the integrity of the membrane is necessary for protection.

DISCUSSION

We have studied the topological action of two eukaryotic peptide sequences in bacterial cells. The hydrophobic 26-residue-long FRHD of the Sendai F protein, situated on the outside of the virus particle, must be transferred, initially, across the bilayer of the endoplasmic reticulum of the host cells. Fused into the bacteriophage f1 coat protein, pIII, this sequence can be moved through the bacterial cytoplasmic membrane into the periplasm. Whereas the carboxyl-terminal hydrophobic sequence that anchors F to the viral envelope functions to stop transfer of the F-pIII hybrid protein and anchors the protein to the membrane. In both cases, behavior in the bacteria reflects the natural biology of these sequences within the eukaryotic cell. The features that govern transport of these sequences across eukaryotic membranes reside within these sequences themselves. This approach of testing discrete sequence elements for stop-transfer activity in bacteria provides a method of general utility for studying the membrane topology of any protein, prokaryotic as well as eukaryotic.

One striking feature of the FRHD of the fusion proteins of paramyxoviruses is the clusters of charged residues either immediately upstream or downstream of it (18–21, 39). For Sendai F protein, six of eight residues just to the carboxyl side of the FRHD are charged (Fig. 1). We thought it was possible that the surrounding charged residues might somehow aid the passage of the FRHD through the membrane. The pDH301-encoded protein lacks this charged sequence, yet it fractionates identically to the pDH221-encoded protein that carries the FRHD plus the surrounding sequence. These surrounding charged sequences, therefore, do not participate in the transport of the FRHD through the membrane.

Examination of the FRHD by hydrophobicity plotting shows this sequence to be one of the more hydrophobic regions of the F protein (Fig. 4). Nonetheless, this sequence clearly is less hydrophobic than the F protein membrane anchor, and it is perhaps somewhat less hydrophobic than the natural pIII membrane anchor. We have compared the hydrophobicity of the F sequences in these hybrid proteins to that of the analogous region of the pND33- and pND34-encoded proteins (5), having, respectively, 3 and 4 repeats of the Leu-Ala-Leu-Val sequence inserted at the same position of the same truncated pIII—i.e., into the *Bam*HI site of pND320 (Fig. 4 Lower). The pND33 protein, having a string of 12 uncharged amino acids, fails to anchor and is secreted to the periplasm, whereas the 16 uncharged residues of the pND34 protein function to stop transfer. The F protein membrane-spanning domain of the pDH409 hybrid protein is clearly far more hydrophobic than the artificial anchor domain of pND34. It is not surprising that it too functions to stop transfer.

On the other hand, the FRHD sequence of pDH301 (and pDH221; data not shown) shows a hydrophobicity similar to the borderline region defined by pND33 and pND34. It would be difficult to predict from this whether the FRHD should anchor or transfer through the membrane. Although the uncharged FRHD is 26 residues long, it shows a degree of hydrophobicity similar to these much shorter hydrophobic sequences. The FRHD sequence contains a somewhat higher percentage of polar residues (e.g., serine, threonine, asparagine, etc.) than sequences that anchor.

How is the FRHD sequence moved through the lipid bilayer? Our results demonstrate that the isolated sequence, the 26 consecutive uncharged residues of the pDH301-encoded protein, have evolved so as to be compatible with passage across the membrane. Part of this evolution might simply reflect the choice and mix of hydrophobic and polar residues, keeping the overall hydrophobicity below some threshold compatible with transfer across the bilayer. Alternatively, at this borderline hydrophobicity, more subtle structural features of this sequence may play a role in its transport across the membrane.

The best evidence to date indicates that the hydrophobicity of this highly conserved FRHD sequence plays an important role in the membrane fusion activity (14, 15, 17, 24–26). The F protein is activated for fusion by a proteolytic cleavage just to the amino-terminal side of the FRHD, yielding two disulfide-linked polypeptides, F₁ and F₂. This cleavage results in a conformational change with exposure of a new hydrophobic binding site (24). It is thought that the exposed

FIG. 4. Hydrophobicity plots of Sendai F protein, pIII, and the local region surrounding the inserted sequence of four constructs (pND33, pDH301, pND34, and pDH409). The parameters of von Heijne (40) were used, except that each data point represents the free energy of transfer (ΔG in kcal/mol; 1 cal = 4.184 J) of a 16- rather than a 21-amino acid segment from a random coil conformation in an aqueous phase to an α -helical conformation in a nonpolar (membrane) phase. The free energy given on the ordinate is the sum of individual contributions for a 16-amino acid segment ending at the residue shown on the abscissa. Hydrophobic sequences plot as minima, and charged sequences plot as maxima. Sequences of interest (signal peptide, membrane anchor, and FRHD) are indicated. The bracketed segment of pIII is shown below for four plasmid-encoded sequences with hydrophobic sequences inserted at the site marked by the arrow.

FRHD can insert into the target lipid bilayer, thereby bringing viral and host membranes together for fusion.

Previous work (5), in which the synthetic hydrophobic segments were inserted at different sites within the pIII sequence, showed that there is a significant effect of position on anchor function. Less hydrophobicity was needed when the anchor was at the carboxyl terminus than when it was inserted at internal positions within the coding sequence. Partial deletion of the natural pIII anchor (27) showed that truncated sequences, some of which were as, or even less, hydrophobic than the FRHD sequence (data not shown), could function to anchor at the carboxyl-terminal site. In the case of Sendai F protein, the activating cleavage moves, in effect, the FRHD sequence from its internal position in the inactive precursor to a terminus, the amino terminus of the F₁ polypeptide. The advantage of being at the end of the protein perhaps increases its propensity for membrane binding, thereby driving the fusion reaction.

On writing this paper, we learn that Paterson and Lamb have fused the FRHD sequence of simian virus 5 to the carboxyl end of a truncated influenza hemagglutinin protein. Expressed in eukaryotic cells, this hybrid protein fractionates with the microsomal membranes and is not extractable by alkali or urea, suggesting an integral association with the membrane (R. G. Paterson and R. A. Lamb, personal communication). As the FRHD of simian virus 5 does not appear to be significantly more hydrophobic than the Sendai sequence (data not shown), this different observation perhaps reflects the different position of the FRHD (terminal versus internal) within the hybrid protein. Having the FRHD in the middle of the hybrid protein reflects its situation during the transfer of the F precursor through the membrane. Positioning it at the end may be more analogous to the cleaved fusion-activated protein.

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