# Determining the Topology of an Integral Membrane Protein



A variety of approaches have been developed for assigning the aqueous domains of integral membrane proteins to either side of a biological membrane. This unit describes three general methods that can be used to solve the topologies of eukaryotic membrane proteins whose primary sequences have been deduced from their corresponding DNA sequences: (1) testing of water-soluble domains for their accessibility to a reagent added to membranes exhibiting a defined orientation (see Basic Protocols 1 and 2), (2) epitope tagging (see Support Protocol), and (3) a genetic approach in which a reporter enzyme fused to various truncated fragments of a membrane protein acts as a sensor of sequences disposed intra- or extracytoplasmically (see Basic Protocol 3).

## STRATEGIC PLANNING

Before initiating an experimental analysis, it is helpful to build a conceptual model of a protein's topology (see Fig. 5.1.2 for a representation of different types of integral membrane proteins). Inspection of the primary sequences of integral membrane proteins usually reveals a pattern of alternating stretches of hydrophilic (water-soluble) and hydrophobic (water-insoluble) amino acids. Very good computer-based approaches are used to illuminate these patterns (Kyte and Doolittle, 1982; Engelman et al., 1986). The results are presented as a graph, in which each amino acid is compared to the hydrophobicity of the amino acids immediately surrounding it in the linear sequence. When visual inspection of the graph reveals a hydrophobic stretch containing at least 18 to 20 amino acids, a segment is identified that is long enough to potentially span the membrane in an α-helical configuration (Deisenhofer et al., 1985). To build this conceptual model, the authors use MacVector software available form Oxford Molecular.

A representative hydropathy analysis of signal peptidase subunit SPC12 (Kalies and Hartmann, 1996) is shown in Figure 5.2.1A. Since this graph reveals an even number of putative transmembrane segments (two) for SPC12, the resulting model predicts that the N and C termini localize to the same side of the membrane. It should be noted that intra- and extracytoplasmic domains are not distinguishable using this analysis; thus, two opposing models for SPC12 topology are compatible with the analysis performed (see Fig. 5.2.1B). A hydropathy plot alone is, therefore, not a predictor of transmembrane orientation, nor is it assured that such a plot will identify all membrane-spanning segments. Experimental evidence is necessary to establish the orientations of integral membrane proteins within the lipid bilayer.

### Protease Digestion

In this experimental approach (see Basic Protocol 1), a protease exhibiting a broad substrate specificity is used as a probe of membrane protein topology. When the enzyme is added to only one side of the target membrane or membrane vesicle, the size of the proteolytic fragment(s) generated reveals the accessible site(s) and hence the orientation across the membrane of the protein segment(s) containing this site or sites. A proteolytic fragment is detected using a specific antibody. Usually, a series of antipeptide antibodies are prepared, such that each hydrophilic domain is recognized by a specific antibody. The number of hydrophilic domains is usually greater than the number of hydrophobic domains by one. For example, SPC12 has two hydrophobic domains and three hydrophilic domains. Thus, a minimum of three antipeptide antibody preparations are needed to probe the hydrophilic domains of SPC12. Sometimes, however, an antipeptide antibody does

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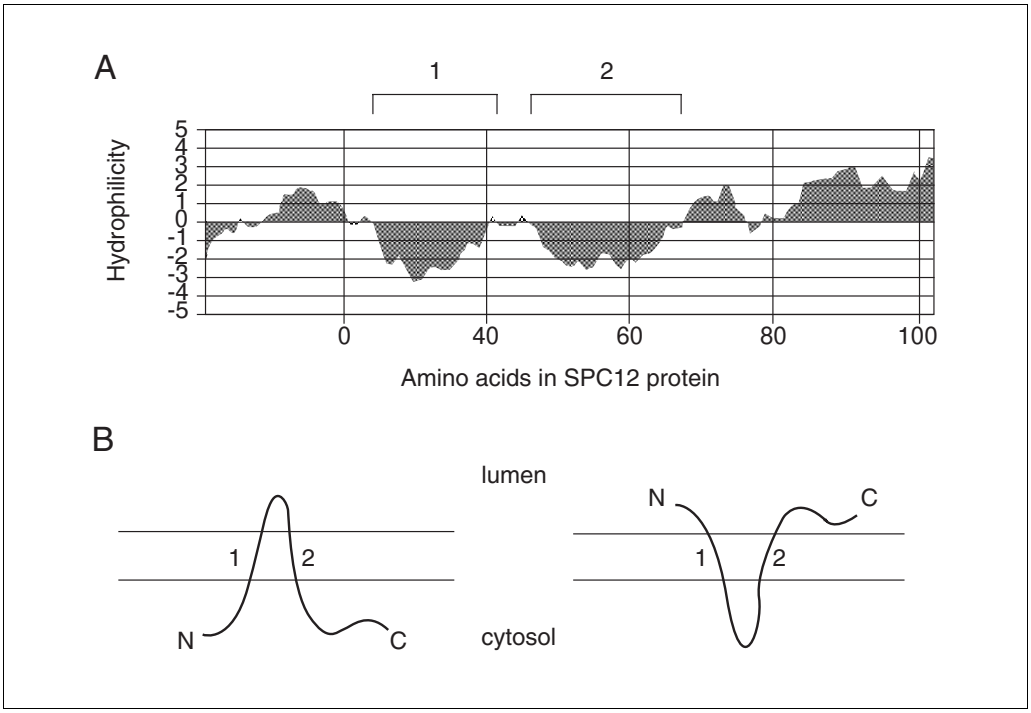


**Figure 5.2.1** (**A**) Hydropathy analysis of SPC12 protein (Kalies and Hartmann, 1996). The data were obtained using the method of Kyte and Doolittle (1982). Hydrophilicity was examined using a window of seven amino acids. The positions of two potential transmembrane segments are shown. (**B**) Conceptualized topologies of SPC12. The data from (A) were used to construct two possible topologies. Hydrophobic segments 1 and 2 are shown spanning the membrane.

not recognize the corresponding peptide sequence in the intact protein; consequently, two or more antipeptide antibody preparations directed against a single hydrophilic domain may be needed.

In addition to this series of antipeptide antibodies, a control antibody is needed that recognizes a luminal protein of the closed membrane system to be analyzed. The orientation of a hydrophilic domain proteolyzed on addition of a protease can then be determined with confidence, providing the luminal protein control is resistant under this condition. A second control uses a mild detergent to solubilize the membranes. Protein sequences localized to both membrane surfaces and to the luminal space are exposed to the protease after detergent addition (Fig. 5.2.2 illustrates these points).

Unusually small hydrophilic domains may be resistant to proteolysis, even if they are exposed to the protease in the absence of detergent. Since addition of detergent often renders the entire protein susceptible, it is not always clear whether a domain that is resistant to proteolytic attack in the absence of detergent is localized to the lumen of the vesicle or just naturally resistant. As discussed below, the accessibility of such domains may be enhanced by using a foreign epitope (or multiple epitopes) inserted into the sequence of a small hydrophilic domain, thereby increasing its size (see Epitope Tagging and Reporter Gene Fusions).

The major advantage of using protease digestion to determine membrane protein topology is that proteins can be examined in their native form without any alterations to their primary sequences, such as those resulting from epitope additions and gene fusions (see Epitope Tagging and Reporter Gene Fusions). The picture derived from protease digestion should therefore reflect the true state of the protein in the cell, especially when the protein is examined in an in vivo expressed form (such as in whole cells or a specific cellular

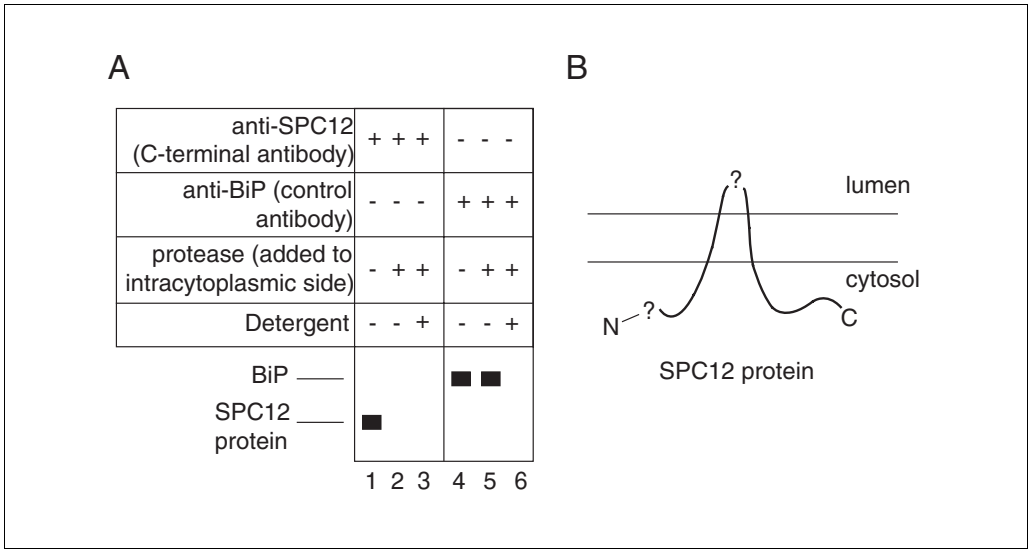


**Figure 5.2.2** (**A**) Idealized immunoblot. The cartoon depicts an immunoblot of proteolyzed microsomes probed with an antibody directed against a C-terminal peptide of SPC12 or control antibodies directed against BiP, a luminal binding protein present in microsomal vesicles (Haas and Wabl, 1984; Bole et al., 1986). Addition or omission of a reagent from the mixture is indicated by + and –, respectively. The positions of BiP and SPC12 are indicated. For further discussion of (A), see Anticipated Results. (**B**) Deduced topology of SPC12's C terminus. The C terminus of SPC12 is presumed to localize to the cytosolic side of the membrane on the basis of the idealized results depicted in (A). The locations of the N terminus and presumed luminal domain of SPC12 cannot be assigned from these data and are therefore indicated with question marks.

organelle). Since membrane proteins are present both at the cell surface and at the surface of intracellular organelles, the literature should be reviewed to pick a procedure for enrichment of the desired membrane from a particular cell type. Examples of protease-digestion studies of membrane proteins residing within isolated organelles and organelle-derived vesicles are described by Graham et al. (1994), Kalies and Hartmann (1996), Kalish et al. (1995), and Li and Shore (1992). Regardless of the procedure chosen, it should produce membranes having a defined orientation, such that polypeptide domains exposed to the lumen, which corresponds to the extracytoplasmic side of the membrane for enriched organelle preparations and microsomal vesicles, are insensitive to the added protease in the absence of detergents.

A disadvantage of the protease digestion approach is that protein fragments must sometimes be identified to assess the topology of a particular region within a membrane protein. Since protein fragments are usually observed by SDS-PAGE (see Fig. 5.2.2) using the protease digestion protocol, small fragments (<2 kDa) may be difficult to detect.

While it is preferable to examine a protein in the membrane in which it resides normally, a procedure may not be available for enrichment of the desired membrane. As an alternative, an in vitro system of enriched microsomal membranes incubated cotranslationally with a membrane protein can be used (Walter and Blobel, 1983; Mize et al., 1986; see *UNIT 11.4*). This in vitro system is useful for examining proteins residing within the endoplasmic reticulum (ER) membrane and proteins transported from the ER to either the lysosome or another compartment of the secretory pathway (such as the cell surface).

### Immunofluorescence Staining

An immunofluorescence staining technique is presented in this unit (see Basic Protocol 2) as a second experimental approach for determining membrane protein topology. In principle, this technique is similar to protease digestion, except that a fluorophore-con-

jugated secondary antibody is employed, instead of a protease, as a probe. A series of antipeptide antibodies is prepared, each recognizing a specific hydrophilic domain of the membrane protein under study. The antipeptide antibodies used for the protease digestion approach may be useful in the immunofluorescence staining approach provided that the antibodies recognize their corresponding epitopes in the membrane protein embedded within the lipid bilayer.

For the immunofluorescence staining protocol, the antibodies are added individually to the surface of an appropriate membrane, which must be closed and exhibit a defined orientation. When the antibody binds to an exposed hydrophilic domain, the antibody can be visualized using a fluorophore-conjugated secondary antibody that emits light detectable by confocal laser scanning microscopy or epifluorescence (see *UNIT 4.3* for a description of these microscopic techniques). Light detected from a bound antibody is evidence that the corresponding hydrophilic domain localizes to the exterior surface of the membrane examined. Protein domains exposed to the inside surface of the plasma membrane can also be probed after membrane permeabilization (Canfield and Levenson, 1993).

As with protease digestion, a major advantage of using the immunofluorescence staining approach is that a protein can be examined in its native form. A further advantage not available using protease digestion is that the orientation of a domain is determined using a microscope instead of detections of proteolytic fragments that may be very small. On the other hand, both immunofluorescence staining and protease digestion approaches suffer from the problem that particularly small hydrophilic domains may be inaccessible to the antibody or protease added. Thus, it may be possible to assign only a subset of the hydrophilic domains to a particular side of the membrane using either approach. Another potential problem with the immunofluorescence staining procedure is that the detergent used to permeabilize the membrane may expose an extracellular domain that is buried within a protein or protein complex. An extracellular domain inaccessible to the antibody in the absence of detergent could therefore be interpreted falsely to localize to the intracellular surface of the membrane.

### Epitope Tagging

In determining membrane protein topology using epitope tagging (see Support Protocol), a foreign epitope is placed within the sequence of a membrane protein. The topology of the domain containing the inserted epitope can then be determined using protease digestion or immunofluorescence staining. The advantage of using a foreign epitope to assess topology is that a single antibody preparation available commercially is employed in lieu of a series of antipeptide antibodies, which can be expensive, time-consuming to prepare, and may not always recognize the intact membrane protein. Furthermore, a small epitope is inserted into a protein's sequence as opposed to fusion of a longer reporter domain, such as in construction of gene fusions (see Reporter Gene Fusion). This small epitope is less likely to alter the native orientation of the protein under study. In addition, multiple epitopes can be inserted into a single hydrophilic domain. The addition of multiple epitopes increases the likelihood that small hydrophilic domains will be accessible to the protease or antibody added. A foreign epitope may, however, inhibit the function of the protein into which it is placed. Since it may be unclear whether epitope insertion disrupts amino acids critical for activity or alters a protein's topology, the results obtained are more reliable when the epitope-tagged protein is shown to be functional in cells or in an in vitro assay.

A general guideline for minimizing interference with the topology of the target protein is to place the epitope ≥15 amino acids from the nearby transmembrane segment(s). This avoids interfering with charged residues that may be important for determining the

orientation of a transmembrane segment (Hartmann et al., 1989). In addition, tagging the N- and C-terminal ends of some membrane proteins should be avoided, since their termini often contain compartment-localization signals (for example, see von Heijne, 1984; Nilsson et al., 1989; Swinkels et al., 1991). However, if it is determined that such signals do not exist at the termini of the target protein, the N and/or C termini are often good sites for epitope tagging, because placement of the epitope at either end does not interrupt the linear sequence of the target protein internally. If the hydrophilic domain to be probed is very small, it may be impossible to place a foreign epitope >15 amino acids from the upstream and/or downstream transmembrane segment. In such instances, one can still place the epitope within the hydrophilic domain; however, caution should be used when interpreting the topological picture derived from study of a very small domain tagged with a foreign epitope.

A variety of epitope tags are available (see Background Information); however, the HA epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984) is recommended because it contains only two nonclustered charges and has been used previously in topological studies (e.g., Canfield and Levenson, 1993). Clusters of negatively charged amino acids have been shown to interfere with the integration of membrane proteins into the ER membrane, especially when the charge cluster is located near the luminal side of a transmembrane segment (Green and Walter, 1992).

Representative DNA fragments used to tag membrane proteins internally and at the N- and C-terminal ends are shown in Figure 5.2.3. These fragments can be synthesized (complementary strands must be prepared) and then inserted into naturally occurring restriction sites or into sites constructed by site-directed mutagenesis. The restriction sites used should be located in sequences corresponding to the protein's hydrophilic domains. To insert these DNA fragments, blunt-end restriction sites should be used when possible, or sites producing sticky ends can be made blunt-ended by standard molecular biological techniques. In addition, the DNA fragments shown in Figure 5.2.3 contain a blunt-end restriction site (*Pvu*II). This restriction site provides a means to introduce a second fragment encoding the HA epitope. Such double-tagged proteins may allow the target protein to be better recognized by anti-HA antibodies than their corresponding single-tagged versions (Canfield et al., 1996). Repetitive insertion of DNA fragments encoding the HA epitope into each new *Pvu*II site makes it possible to create proteins tagged multiple times within a single hydrophilic domain. It should be noted, however, that increasing the size of a particular domain significantly may render the protein nonfunctional.

### Reporter Gene Fusions

A genetic approach for determining membrane protein topology is also available (see Basic Protocol 3). A series of gene fusions are constructed in which an enzyme (reporter moiety) is joined to various truncated fragments of an integral membrane protein (Fig. 5.2.4). The enzyme used for a reporter moiety is chosen because it exhibits activity only when placed on one side of the membrane. On expression of the fusions in vivo, measurements of enzyme activity indicate the orientation of the domains that contain the fusion joints (Fig. 5.2.5). This approach has been developed to examine proteins inserted into the membrane of the ER in the yeast *Saccharomyces cerevisiae* (Sengstag et al., 1990). However, numerous membrane proteins located throughout the secretory pathway and on the surfaces of cells contain signals for targeting to the ER membrane. These proteins are therefore amenable to topological analysis on integration within the ER membrane prior to trafficking to their ultimate destinations.

To avoid interfering with integration of the target protein, the joint connecting the membrane protein fragment and the reporter moiety should be placed ≥15 amino acids

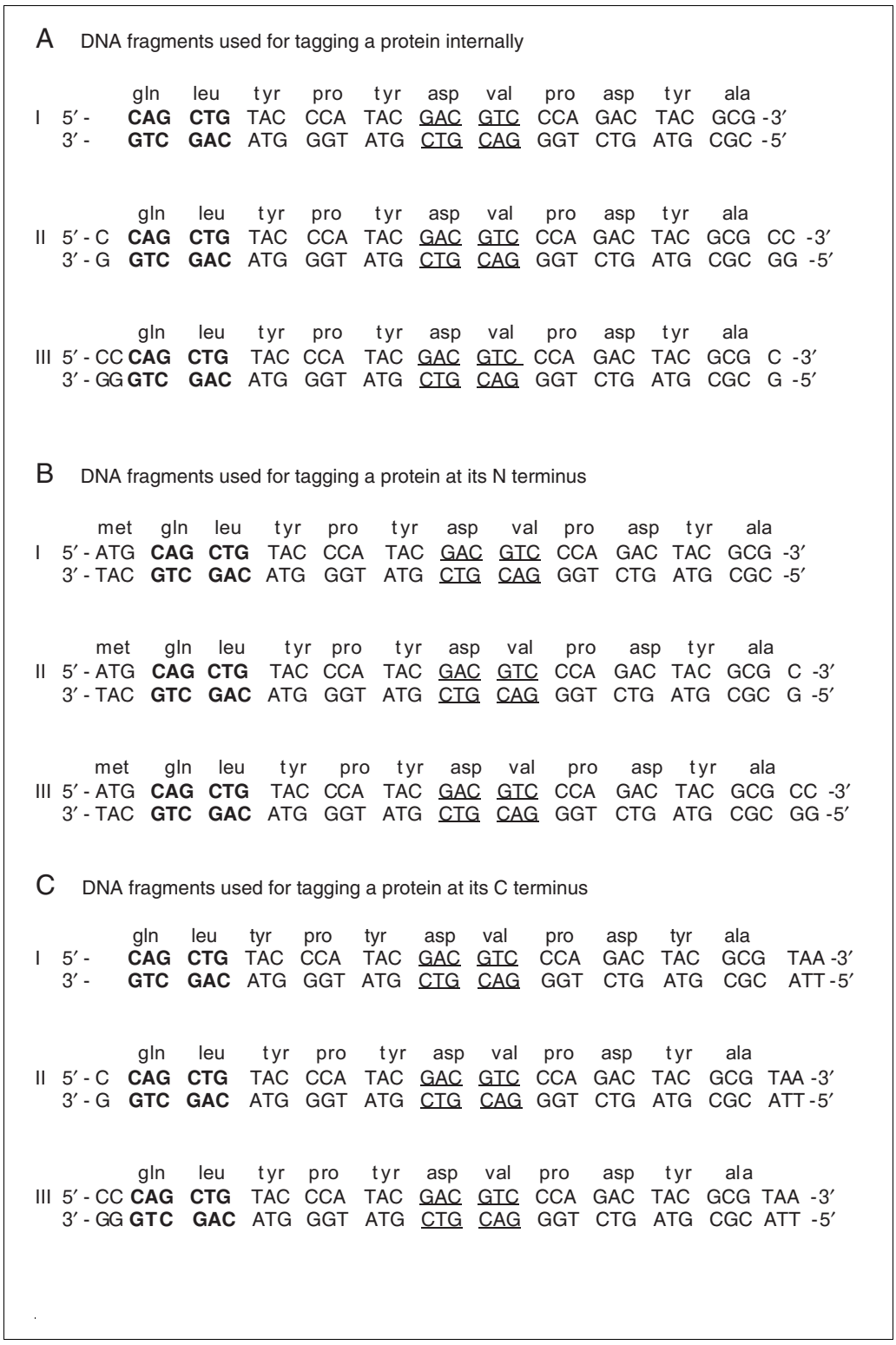


**Figure 5.2.3** Examples of DNA fragments used for tagging proteins with the HA epitope. Use of fragments I, II, and III allow in-frame fusions to any reading frame internally (**A**), at the N terminus (**B**), or at the C terminus (**C**). If the translation initiation and termination codons endogenous to the gene of interest are used, the ATG codons shown in (B) and TAA codons shown in (C) can be eliminated from the fragments depicted here. *Pvu*II (bold) and *Aat*II (underlined) restriction sites are indicated.

from the upstream transmembrane segment. When probing a hydrophilic domain containing <15 amino acids, it is acceptable to place the fusion joint after the first 3 to 5 amino acids of the following hydrophobic stretch, if one exists, in order to avoid disrupting charged residues that may be critical for orienting the upstream transmembrane segment (an example of this placement within a downstream transmembrane segment is shown in Figure 5.2.6). If, on the other hand, the small domain being probed lies at the protein's C terminus, the fusion joint should be placed at the C-terminal end of the protein.

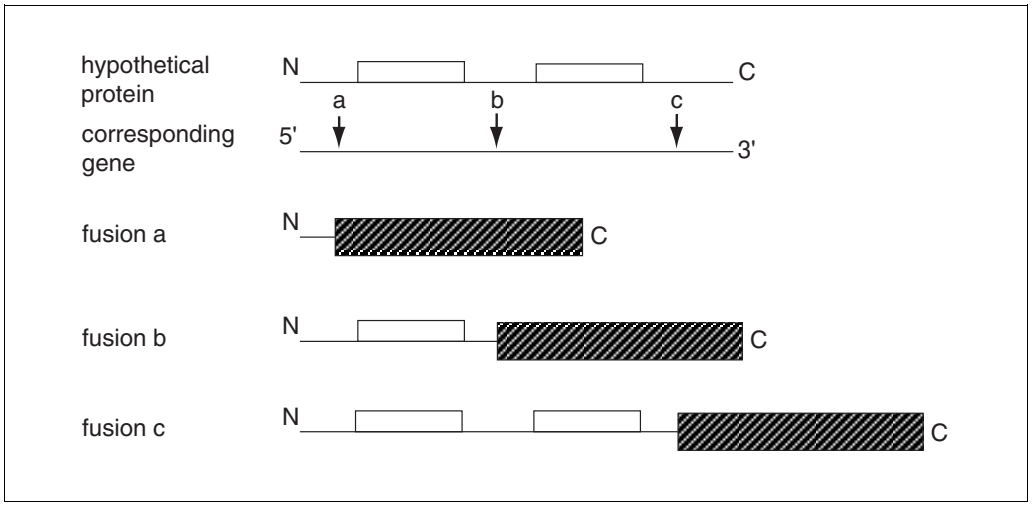


**Figure 5.2.4** Placement of fusion joints in an integral membrane protein. Putative transmembrane segments are indicated by open boxes. The positions of the fusion joints to be constructed in the corresponding gene are indicated by arrows and denoted a, b, and c. A series of fusions of the invertase fragment-histidinol dehydrogenase (inv-HD) sequence (striped box) to N-terminal fragments of the hypothetical protein are indicated as fusions a, b, and c.

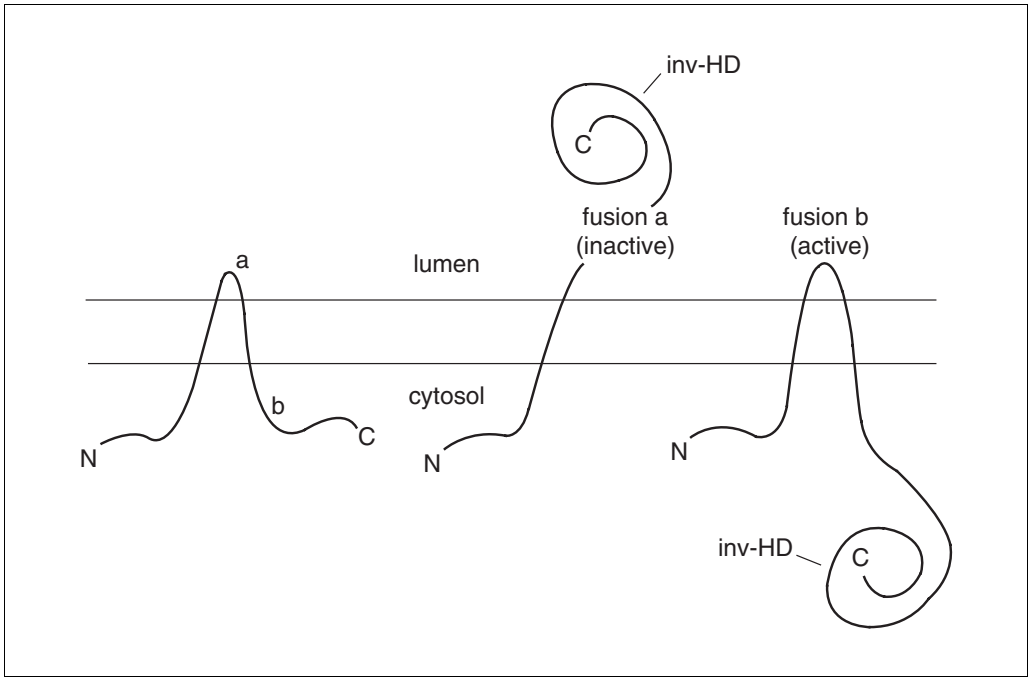


**Figure 5.2.5** Fusion approach to determining membrane protein topology. The fusion of inv-HD at positions a and b in a hypothetical protein are indicated. Fusion a lacks HD activity due to the translocation of HD to the luminal side of the membrane. Fusion b exhibits HD activity due to the presence of HD in the cytosol.

## Selecting a Method

A number of factors govern the selection of a protocol best suited for assessing the topology of a particular protein. Since protease digestion and immunofluorescence staining are used to probe a full-length protein, as opposed to truncated fragments, results obtained from these approaches may be more reliable, although problems associated with accessibility to the protease or antibody used can occur. Use of a foreign epitope to assess membrane protein topology can also yield very reliable results, provided the inserted epitope does not inhibit function of the target protein. Because of its simplicity, however, the reporter fusion approach is often employed as a way to get a general picture. As this approach involves fusion of a large foreign domain to N-terminal fragments, the topological assignments should be augmented with measurements of at least a few hydrophilic regions in the intact functional protein to determine whether different approaches yield the same topological picture. By complementing the reporter fusion analysis with one of the first two approaches, the derived map should provide a reasonably accurate assessment of the protein's topology. An example of this complementary approach is described by Lai et al. (1996).

***BASIC PROTOCOL 1***

## PROTEASE DIGESTION

This protocol uses ER-derived vesicles from canine pancreas. These microsomes are purified according to the method of Walter and Blobel (1983) and exhibit a cytoplasmic-side-out orientation. Digestion of a hydrophilic domain present on the surface of microsomal vesicles therefore indicates that the exposed region is disposed to the cytosolic side of the membrane. This protocol uses immunoblotting to examine unlabeled proteins present in these membranes, although radiolabeled proteins integrated in vitro can also

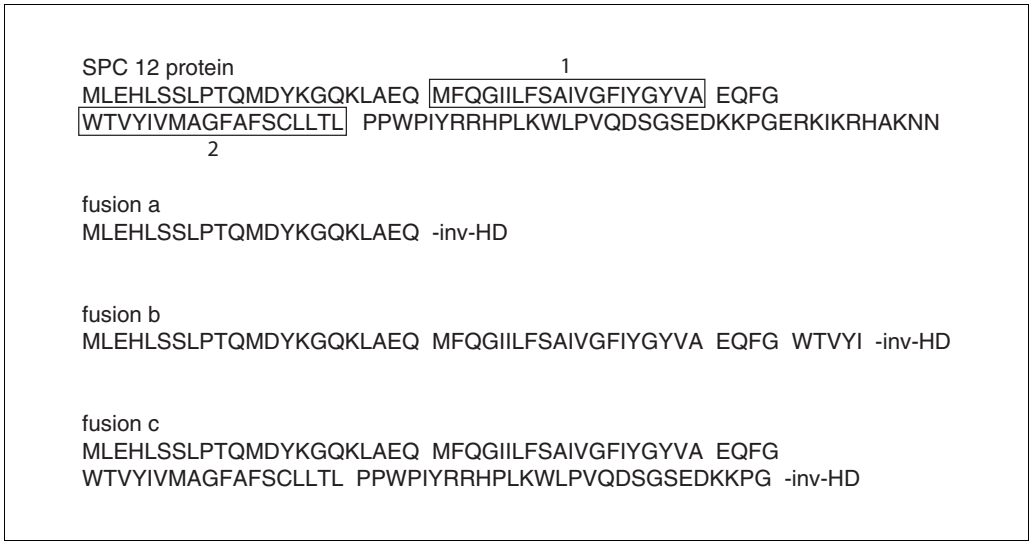


**Figure 5.2.6** Positioning of fusion joints in SPC12 protein. The inv-HD reporter was placed at three positions in the SPC12 protein to discern the orientation of the three hydrophilic domains flanking hydrophobic segments 1 and 2 (open boxes). Note that the fusion joint of fusion c is placed >15 amino acids from the upstream hydrophobic segment. However, since the hydrophilic domain located between hydrophobic segments 1 and 2 consists of only 4 amino acids (EQFG), it is not possible to place the fusion joint more than 15 amino acids from hydrophobic segment 1 without capturing most of hydrophobic segment 2 in fusion b. Therefore, the fusion joint is positioned such that only 5 amino acids of hydrophobic segment 2 are present in fusion b. In placing the fusion joint at this position, the charged residues immediately following hydrophobic segment 1 are left undisturbed. Fusion a lacks a putative transmembrane segment and will remain cytosolic regardless of the topology of the N terminus of intact SPC12 protein. One exception to this is that, if the polypeptide chain contains an N-terminal signal sequence (von Heijne, 1984), fusion a would probably be transported to the ER lumen.

be studied (Walter and Blobel, 1983; Mize et al., 1986; see *UNIT 11.4* for analysis of protein translocation into canine microsomes). The protocol can also be adapted to microsomes prepared from metabolically labeled cells (*UNIT 7.1*; Bonnerot et al., 1994). As a control to determine whether the target protein is inherently sensitive to the protease used, proteolyzed membranes are compared to membranes solubilized in a mild detergent, such as Triton X-100, prior to protease addition.

In this protocol, proteinase K is employed as a representative protease due to its broad substrate specificity. In addition, proteinase K is active under the buffer conditions described (as are many other proteases), and proteinase K retains activity in the presence of Triton X-100. The buffer also contains sucrose to help maintain the integrity of membrane vesicles. Another control, which tests for the presence of intact membranes, examines a luminal protein native to the membrane vesicles used. Provided the membranes are intact, the luminal protein control should be resistant to proteinase K in the absence of detergent. Examples of ER proteins used for control purposes are TRAPβ (Kalies and Hartmann, 1996) and BiP (Mullins et al., 1995).

### *Materials*

Canine pancreatic microsomal membranes (see *UNIT 11.4*)
Magnesium/sucrose/BSA (MSB) buffer (see recipe)
20% (w/v) Triton X-100
10 mg/ml proteinase K (see recipe)
100% (w/v) trichloroacetic acid (TCA) (see recipe)
2× SDS sample buffer (*APPENDIX 2A*)
Antibodies directed against a series of peptides corresponding to specific hydrophilic regions of the target protein
Control antibodies directed against a luminal protein or a luminal domain of a membrane protein in the membrane system to be analyzed

Additional reagents and equipment for preparing canine pancreatic microsomes (*UNIT 11.4*), separating proteins by SDS-PAGE (*UNIT 6.1*), and detecting proteins by immunoblotting (*UNIT 6.2*)

### *Prepare microsomal membranes*

1. Purify microsomal membranes according to the method described in *UNIT 11.4*.

   *Microsomes are stored at −80°C at a final concentration of 50 $A_{280}$ units per ml. One equivalent is defined as 1 μl of this mixture. When stored under these conditions, microsomes are stable ≥1 year.*

   *Be sure that inhibitors of the protease to be used are omitted from the original membrane preparation (such as PMSF in the case of proteinase K; see APPENDIX 1B). If protease inhibitor is present, wash the membranes by centrifuging 20 min at ~100,000 × g, 4°C. Suspend the pellet in MSB buffer at the same concentration by pipetting up and down in a plastic tip.*

2. Slowly thaw ~50 μl of microsomes by placing a tube containing the microsomes in ice.

   *This amount of membranes is needed for examinations that use one antipeptide antibody and a control antibody. An additional 50-μl aliquot is needed for each additional examination.*

3. Prepare ten samples, each containing 5 μl (5 equivalents) rough microsomes diluted with MSB buffer to a final volume of 20 μl in microcentrifuge tubes.

   *Steps 3 to 8 should be performed using tubes kept in ice. Polypropylene microcentrifuge tubes have been found to work satisfactorily.*

4. Add 1 µl of 20% (w/v) Triton X-100 to five of the tubes and mix by pipetting the solution up and down.

   *These detergent-treated aliquots serve as controls to demonstrate that the target protein is inherently sensitive to the protease used. The five tubes lacking Triton X-100 are sample tubes and the five tubes containing Triton X-100 are control tubes.*

***Perform protease digestion***

5. Add 10 mg/ml proteinase K to four of the sample tubes and four of the control tubes to yield final concentrations of 1, 20, 100, and 500 µg/ml.

   *The amount of protease is varied to find the concentration which allows good digestion of the target protein yet is low enough that the protease does not destroy the membrane structure (thus making the membrane permeable to the protease added). Protease should be omitted from the fifth tube in the sample and control sets.*

6. Mix samples by pipetting the solutions up and down a few times and incubate 30 min.

7. Stop proteolysis by adding 100% (w/v) TCA to a final concentration of 15%.

***Analyze protein digests***

8. Incubate all ten tubes 15 min on ice and collect TCA pellets by microcentrifuging 5 min at ~10,000 rpm (8,000 × *g*), room temperature.

9. Remove supernatant and suspend the pellets in 10 µl of 2× SDS sample buffer and 10 µl $H_2O$ by vortex mixing at room temperature.

   *Be sure that all of the pellet has been suspended. The mixtures should be blue because of bromphenol blue, which is present in the sample buffer. If the tubes are yellow (because of high acidity), 1-µl aliquots of 1 M Tris base can be added successively and then mixed until the mixture turns blue.*

10. Load 10 µl from each tube into sample wells of a SDS-PAGE gel.

    *The lanes should be organized as follows. The first five lanes contain one-half of the mixture from the five sample tubes, and the next five lanes contain one-half of the mixture from the five control tubes. The next five lanes contain one-half of the mixture from the five sample tubes, and the final five lanes contain one-half of the mixture from the five control tubes.*

    *A 12.5% polyacrylamide gel can be used to identify protein fragments of ~2 to 30 kDa, whereas a 7% acrylamide gel can be used to identify protein fragments of 30 to 100 kDa.*

11. Analyze samples using SDS-PAGE (*UNIT 6.1*).

12. Examine the separated protein fragments by immunoblotting (*UNIT 6.2*), using one of the antipeptide antibodies and the control antibody directed against a luminal protein or a luminal domain of a membrane protein in the membrane system to be analyzed.

    *The blot should be cut in half. The gel portion containing the first ten lanes is to be immunoblotted with an antibody directed against the target protein. The gel portion containing the second group of ten lanes should be subjected to immunoblotting using an antibody directed against the luminal protein control. The data should be interpreted by following examples described in Figure 5.2.2A and Anticipated Results.*

13. Repeat steps 3 to 12 using each of the antipeptide antibodies and the control antibody.

## IMMUNOFLUORESCENCE STAINING

This protocol illustrates use of an immunofluorescence technique to probe the topology of membrane proteins. The protocol is designed specifically to examine proteins confined to the plasma membranes of cells lacking an outer cell wall, such as cultured mammalian cells. This protocol has been optimized for using human embryonic kidney (HEK) 293 cells, although other cultured cell lines can be used with only minor modifications (noted below). These cells can be grown on glass coverslips and permeabilized with low concentrations of a mild detergent (Nonidet P-40 is used here), which allows antibodies access to the cytoplasmic compartment. The permeabilized cells and a second nonpermeabilized cell preparation are incubated with an antipeptide antibody followed by incubation with a fluorophore-conjugated secondary antibody. Cells are then examined by fluorescence microscopy (*UNIT 4.2*). As a control, cells expressing a plasma membrane protein of known topology should be examined using antibodies directed to a cytosolic domain and an extracytoplasmic domain.

This approach can employ primary antibodies directed against peptides corresponding to the membrane protein under study, such as the antipeptide antibodies described in Basic Protocol 1. However, antipeptide antibodies are found often not to recognize their corresponding epitopes within a native membrane protein (Carrasco et al., 1986). The procedure described here therefore uses a membrane protein that has been tagged with the HA epitope (see Support Protocol for methods regarding epitope tagging). Use of the HA epitope can minimize cost and time associated with the preparation of antipeptide antibodies. The primary and secondary antibodies used in this protocol are the anti-HA epitope mouse monoclonal antibody 12CA5 and a rhodamine-conjugated rabbit anti-mouse IgG, respectively, but other combinations of antibodies can be used as well (Canfield and Levenson, 1993).

### *Materials*

HEK 293 cells (ATCC #CRL 1573)
4% (w/v) paraformaldehyde (see recipe)
Nonidet P-40/goat serum/BSA (NGB) solution (see recipe)
Anti-HA mouse monoclonal antibody 12CA5 (Boehringer Mannheim)
Rhodamine-conjugated rabbit anti-mouse immunoglobulin G (IgG)
DMEM/FBS/HEPES (DFH) solution (see recipe)
Fluoromount G mounting medium (Fisher)

6-well tissue culture plates
Glass coverslips, 22-mm diameter

Additional reagents and equipment for immunofluorescence staining of fixed mammalian cells (*UNIT 4.3*), epifluorescence (*UNIT 4.2*) or confocal laser microscopy, and growing cultured mammalian cells (*UNIT 1.1*)

1. Seed HEK 293 cells stably expressing the polypeptide of interest on glass coverslips and grow 2 to 3 days at 37°C in 6-well plates before processing for immunofluorescence.

   *Alternatively, transient transfections can be performed on cells grown on glass coverslips in 6-well plates (UNIT 1.1). Cells should be handled using typical aseptic technique required for growing cells in culture (UNIT 1.3).*

   *The glass coverslips should be cleaned thoroughly using a strong detergent and washed extensively with distilled water.*

   *Duplicate cultures are needed in order to provide sets to be examined in the absence and presence of permeabilizing agents and in the absence or presence of the polypeptide of*

*interest. A single 6-well plate thus provides a triplicate analysis of cells to be treated with permeabilizing agents and containing the polypeptide of interest and a triplicate analysis of cells treated with permeabilizing agents and lacking the polypeptide of interest. A distinct plate provides a triplicate analysis for the set not treated with permeabilizing agents and either containing or not containing the polypeptide of interest (see below).*

*All steps except the antibody incubations can easily be carried out in 6-well tissue culture plates, using volumes of 2 to 3 ml per well for incubations and washes.*

*Methods for HA tagging, and testing for function of tagged proteins in transiently transfected HEK 293 cells are described in the Support Protocol.*

2. For the permeabilized set, aspirate culture medium and wash coverslips once in PBS. Replace PBS with 4% paraformaldehyde (2 to 3 ml) and fix 10 min at room temperature.
3. Aspirate fixing reagent and rinse cells three times with PBS. Permeabilize and block cells in NGB solution (2 to 3 ml) for 15 min at room temperature.
4. Dilute monoclonal antibody 12CA5 to 1 µg/ml in NGB. Centrifuge antibody dilution 2 min at 8000 × *g* to remove aggregates.

   *Although this antibody is supplied at a specified concentration, different batches appear to have different characteristics, and it may be necessary for individual users to optimize their antibody dilutions.*

5. Place coverslips cell-side-up on Parafilm and immediately add primary antibody (100 µl per coverslip). Incubate 1 hr at room temperature in a covered, humidified chamber.

   *A small petri dish or any small covered container will serve as a humidified chamber.*

   *For more tightly adherent cells, the volume of antibody used can be reduced further as follows. Pipet antibody (50 µl) directly onto Parafilm. Place coverslips on droplet, cell-side-down.*

6. Return coverslips to NGB (2 to 3 ml) in the 6-well plate. Wash three times with PBS (10 min each wash) and once with NGB.
7. Incubate cells 1 hr at room temperature in NGB solution (2 to 3 ml) containing a rhodamine-conjugated rabbit anti-mouse IgG.

   *The secondary antibody should be diluted according to the supplier's recommendations. Optimization of antibody dilution may be necessary.*

8. Return coverslips to NGB solution and wash three times with PBS.
9. Dip coverslips in distilled water and mount on slides using Fluoromount-G (15 µl per slide).
10. For the nonpermeabilized set, replace growth medium with DFH solution and chill 15 min to 4°C.

    *These cells provide the set that is nonpermeable in the presence of primary antibody. These steps can most conveniently be performed concurrently with processing of permeabilized cells.*

11. Incubate with monoclonal antibody 12CA5 (2 µg/ml) for 1 hr in a humidified chamber at 4°C as described in step 5.

    *Note that a higher concentration of antibody is used for this low-temperature incubation.*

12. Wash three times with cold DFH and once with cold PBS.

13. Fix, permeabilize, and incubate "nonpermeabilized" cells with secondary antibody, as described in steps 2 to 3 and 7 to 9.

    *The nonpermeabilized set is permeabilized immediately before addition of secondary antibody. This step serves not only to bind the secondary antibody to the primary antibody but also to determine whether the secondary antibody binds nonspecifically to either surface of the plasma membrane.*

14. Examine permeabilized and nonpermeabilized cells using epifluorescence or confocal laser scanning microscopy.

    *Fluorescence staining of only the permeabilized set is suggestive of an antibody bound to a cytosolic domain, whereas fluorescence appearing in both the permeabilized and nonpermeabilized sets reveals a domain placed at the extracytoplasmic side of the plasma membrane. A range from 5% to 25% of the cells on a particular coverslip may be stained well.*

    *The control cells lacking the polypeptide of interest are needed because one difficulty associated with the use of 12CA5 is that it is not absolutely specific for the HA epitope. Cross-reactivity is seen both on immunoblots and by immunofluorescence in nontransfected or nontagged cells.The resulting background may decrease the sensitivity with which the transfected, tagged polypeptide can be visualized. If the background signal is too high to detect the protein of interest, even after diluting the primary and secondary antibodies further than that described above, try other anti-HA antibody preparations such as those available from BAbCO. Note, however, that some of these antibodies may not recognize HA tags placed internally within the protein sequence.*

## EPITOPE TAGGING

***SUPPORT PROTOCOL***

In this protocol, a series of molecular biological manipulations are used to place a foreign epitope into a hydrophilic region of a membrane protein. Integration of the expressed protein leads to placement of the epitope on one side of the membrane. The orientation of the tagged domain can be assessed by monitoring the accessibility of the epitope to a protease (see Basic Protocol 1) or to its cognate antibody (see Basic Protocol 2).

The HA epitope, YPYDVPDYA, is recommended for use in this protocol because it contains only two charged amino acids. As shown in Figure 5.2.3, a set of three DNA fragments (differing by addition of 0, 1, or 2 base pairs at one or both ends) can be used to place the HA epitope internally within the sequence of a protein, N-terminally, or C-terminally. The fragments are inserted into a blunt-end restriction site occurring naturally within the protein's gene or into a restriction site constructed by site-directed mutagenesis (see *APPENDIX 3*). Depending on the reading frame of the site into which the epitope is inserted, only one of the three DNA fragments is needed to permit in-frame fusion between the membrane protein sequence and the epitope at its N and/or C termini. These fragments also contain the blunt-end *Pvu*II restriction site. This site provides a convenient place to insert a second fragment encoding the HA epitope, which is used if the single tag is found not to present the membrane protein adequately to the added antibody. Alternatively, DNA fragments containing a blunt-end restriction site other than *Pvu*II, such as *Eco*RV, can be synthesized if this *Pvu*II site is not unique on the plasmid containing the gene of interest. The DNA sequence encoding the HA epitope depicted in Figure 5.2.3 also contains an *Aat*II restriction site that can be used to verify insertion of the fragment into the plasmid used. It is usually necessary to verify orientation of the inserted fragment by DNA sequencing. This is not the only way to introduce an epitope into a membrane protein. A DNA sequence encoding the epitope can be introduced by site-directed mutagenesis.

Having introduced a DNA fragment encoding the HA epitope into a cloned gene, the tagged protein should be tested for function. If the tagged protein exhibits normal functional properties, then it is likely that the epitope introduced has not altered the overall

structure of the protein. To test for function, many types of assays can be performed. The type of assay used is dependent on the particular protein under study. For example, the HA-tagged rodent Na,K-ATPase α subunit can be tested for its ability to confer ouabain resistance to HEK 293 cells transfected with the tagged DNA construct (see Basic Protocol 2; Canfield et al., 1996). A tagged protein can be introduced into yeast cells, testing for complementation of a mutant lacking that protein or an evolutionarily conserved protein (Kurihara and Silver, 1993). Alternatively, a tagged protein may be expressed to high levels, enriched, then tested for activity using an in vitro assay.

***Materials***

TE buffer (*APPENDIX 2A*)
Plasmid DNA encoding the protein of interest
*E. coli* cells to be transformed

Additional reagents and equipment for synthesizing oligonucleotides (*APPENDIX 3*), ligating DNA (*APPENDIX 3*), transforming *E. coli* (*APPENDIX 3*), isolating plasmid DNA from *E. coli* (*APPENDIX 3*), identifying plasmids by restriction endonuclease digestion (*APPENDIX 3*), and sequencing oligonucleotides (*APPENDIX 3*)

1. Determine whether fragment I, II, or III (Fig. 5.2.3) is needed to produce an in-frame fusion between the N- and/or C-terminal ends of the HA epitope and the target protein.

2. Synthesize the appropriate DNA fragment(s) identified from step 1.

   *Complementary oligonucleotides should be synthesized.*

3. Mix two complementary DNA strands together, each to a final concentration of 1 $A_{260}$ unit per 50 µl in TE buffer.

   *Oligonucleotides are usually synthesized with their 5′ ends unphosphorylated. The oligonucleotides used should be left unphosphorylated to prevent insertion of multiple oligonucleotides into the linearized plasmid.*

4. Anneal the strands by heating the solution 5 min at 70°C and then letting the solution cool in air at room temperature.

5. Mix 5 µl of the oligonucleotide solution with 0.1 µg of linearized plasmid DNA encoding the protein of interest, ligate the mixture in 10 µl of ligation buffer, and transform *E. coli* with the ligation mixture (*APPENDIX 3*).

   *The DNA should be linearized at a restriction site contained within a sequence corresponding to the hydrophilic domain to be probed. If such a restriction site does not exist, one can be introduced by site-directed mutagenesis (as illustrated below, blunt-end sites are preferable). If possible, the epitope should be placed ≥15 amino acids from the nearby transmembrane segment(s). If the restriction site used produces sticky ends, the site should be made blunt-ended using standard molecular biological manipulations. To minimize religation of the plasmid without the DNA fragment to be inserted, the ligation mixture should be treated with the restriction enzyme used to produce the blunt end, provided a restriction enzyme producing blunt ends was used.*

   *The above-described ratio of fragment and linear plasmid is acceptable in many instances. However, sometimes this ratio may need to be varied in order to achieve efficient ligation of the fragment to the plasmid. Try increasing the concentration of oligonucleotides from 1 $A_{260}$ unit to 2, 5, and 10 $A_{260}$ units per 50 µl in TE buffer (step 3).*

   *Any of the E. coli strains commonly used for plasmid transformations is suitable, such as HB101 and MC1061.*

6. Isolate plasmids from individual *E. coli* transformants and identify plasmids containing the insert using restriction analysis (*APPENDIX 3*).

*If a fragment shown in Figure 5.2.3 is synthesized, restriction enzymes AatII and PvuII are useful for identifying the desired construct.*

7. Determine the DNA sequence of the fragment inserted and residues surrounding this fragment to ensure correct orientation and in-frame fusion.

8. Express the epitope-tagged protein in an appropriate cell and test for its function. Examine its topology using Basic Protocols 1 or 2.

   *If the tagged protein is not recognized by antibodies directed against the HA epitope, double-HA-tagged proteins can be constructed by inserting the appropriate DNA fragment (Fig. 5.2.3) into the PvuII site of the construct tagged with only one HA epitope. Successive insertions into each new PvuII site will produce proteins tagged with increasing numbers of HA epitopes.*

   *Plasmid DNA can be stored indefinitely in TE buffer at −20°C and should be introduced into the cell examined prior to use.*

## REPORTER GENE FUSIONS

***BASIC PROTOCOL 3***

In this protocol, a series of gene fusions is constructed in which a reporter moiety is fused to various truncated fragments of a target membrane protein. To construct these fusions, DNA fragments encoding a series of N-terminal fragments of a membrane protein should be synthesized by the polymerase chain reaction (PCR; see *APPENDIX 3*). For each PCR amplification, two primers are required: one corresponding to a sequence upstream of the promoter of the relevant gene and one corresponding to sequences at the desired fusion joint. Only one upstream primer is required, whereas a downstream primer corresponding to each of the fusion joints is usually needed. For construction of PCR fragments, the upstream primer contains a *Bam*HI site or a site compatible with the *Bam*HI sticky end, such as *Bgl*II or *Bcl*I. The downstream primer contains a *Xho*I site or a site compatible with *Xho*I, such as *Sal*I. Compatible sites are needed when the DNA fragment to be inserted contains an internal *Bam*HI or *Xho*I site. To ensure that the reading frame is maintained across the fusion joint, the *Xho*I site in the downstream primer is placed in the following reading frame: C TCG AG (where TCG encodes an in-frame serine).

A vector that can be used for expression of fusions in yeast is pA189invHD (Green and Walter, 1992; Fig. 5.2.7). pA189invHD is used for construction of gene fusions encoding the C-terminal moiety histidinol dehydrogenase (HD). HD is a cytoplasmic enzyme that is enzymatically inactive when fused to a luminal domain of a membrane protein inserted into the ER membrane. This shuttle vector contains the 2μm DNA fragment for replication in yeast and the ColE1 replication origin for replication in *E. coli*. pA189invHD can be selected in yeast cells containing a mutation in the *URA3* gene due to the presence of plasmid-borne *URA3*. The vector can also be maintained in *E. coli*, as it confers ampicillin resistance. pA189invHD contains a single *Bam*HI site and a single *Xho*I site. Restriction of pA189invHD with *Bam*HI and *Xho*I generates two DNA fragments (13 kb and 0.8 kb). The larger of these fragments contains the selectable markers and origins of replication described above. The smaller fragment encodes a portion of arginine permease, whose topology was analyzed previously using this vector system (Green and Walter, 1992). The fragment encoding arginine permease is therefore replaced with PCR-amplified fragments encoding N-terminal truncations of the membrane protein to be examined. For examining the topology of a protein without its native promoter, the yeast *ADH1* promoter (Bennetzen and Hall, 1982) is included on pA189invHD. When using the *ADH1* promoter, the upstream primer should contain a *Bam*HI site in front of the initiation codon for the membrane protein. For efficient expression from the *ADH1* promoter in pA189invHD, it is important that no ATG codon is present between the *Bam*HI site and the initiation codon.

### *Materials*

Reporter plasmid (Fig. 5.2.7): pA189invHD (available from Neil Green, Vanderbilt University)

*S. cerevisiae* strain FC2-12B (*MATα trp1-1 leu2-1 ura3-52 his4-401 HOL1-1 can1-1*; available from Neil Green, Vanderbilt University)

SD +HIS agar plates (see recipe)

SD +HOL agar plates (see recipe)

Thermocycler

Additional reagents and equipment for the polymerase chain reaction (PCR; *APPENDIX 3*), agarose gel electrophoresis (*APPENDIX 3*), restriction endonuclease digestion (*APPENDIX 3*), and transformation of *E. coli* and *S. cerevisiae* (*APPENDIX 3*)

### *Construct gene fusions*

1. Amplify by PCR a series of DNA fragments encoding truncations of a membrane protein, using appropriate primers.

   *The DNA primers used in the PCR amplification can be synthesized by one of a number of companies offering services in oligonucleotide synthesis. The upstream primer should*

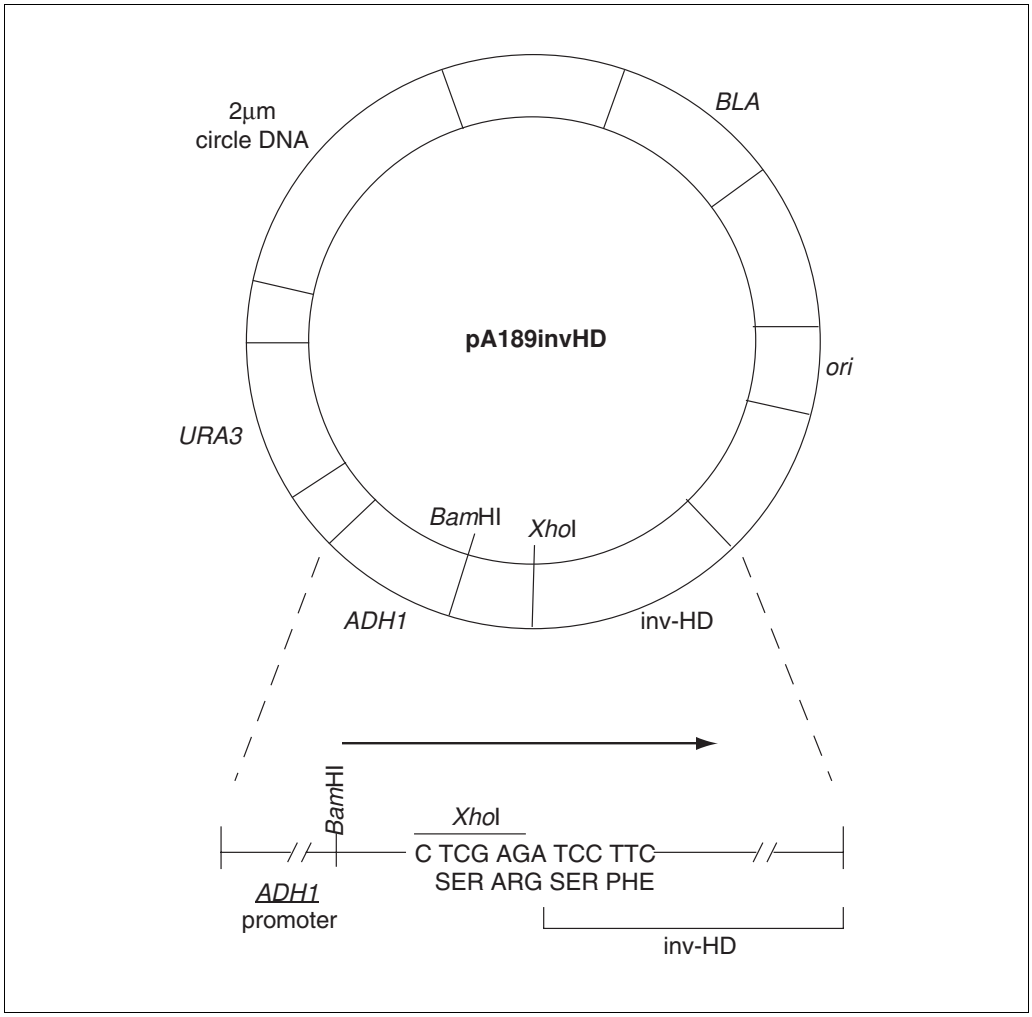


**Figure 5.2.7** Gene fusion vector pA189invHD. The direction of transcription is indicated by an arrow. Abbreviations: *BLA*, β-lactamase gene; *ADH1*, alcohol dehydrogenase gene promoter; *URA3*, orotidine-5′-phosphate decarboxylase gene; ori, ColE1 replication origin; inv-HD, fusion of invertase fragment-histidinol dehydrogenase genes.

*contain a 5′ BamHI site (or compatible site) followed by at least 20 nucleotides complementary to the DNA sequence to be amplified. The downstream primer should contain a 5′ XhoI site (or compatible site) followed by at least 20 nucleotides from the reverse strand of the DNA sequence to be amplified. The BamHI site should be placed immediately before the initiation codon if the ADH1 promoter of pA189invHD is to be used or before the promoter of the gene to be studied. The XhoI site should correspond to a site in a hydrophilic domain of the membrane protein studied. It is also recommended that the primers contain 2 to 3 nucleotides at the 5′ end of the restriction site for efficient cutting by the restriction enzyme after PCR amplification.*

2. Purify the amplified fragments by agarose gel electrophoresis and digest with *Bam*HI and *Xho*I.

   *Overnight restrictions are sometimes needed for efficient cutting of XhoI sites located near the ends of DNA fragments.*

3. Digest the reporter plasmid pA189invHD with *Bam*HI and *Xho*I.

4. Purify the larger, 13-kb DNA fragment produced from the restriction digestion by agarose gel electrophoresis (see *APPENDIX 3*). Ligate the 13-kb fragment to each of the fragments produced in step 2.

   *The smaller fragment (0.8 kb) encodes a part of arginine permease and should be discarded.*

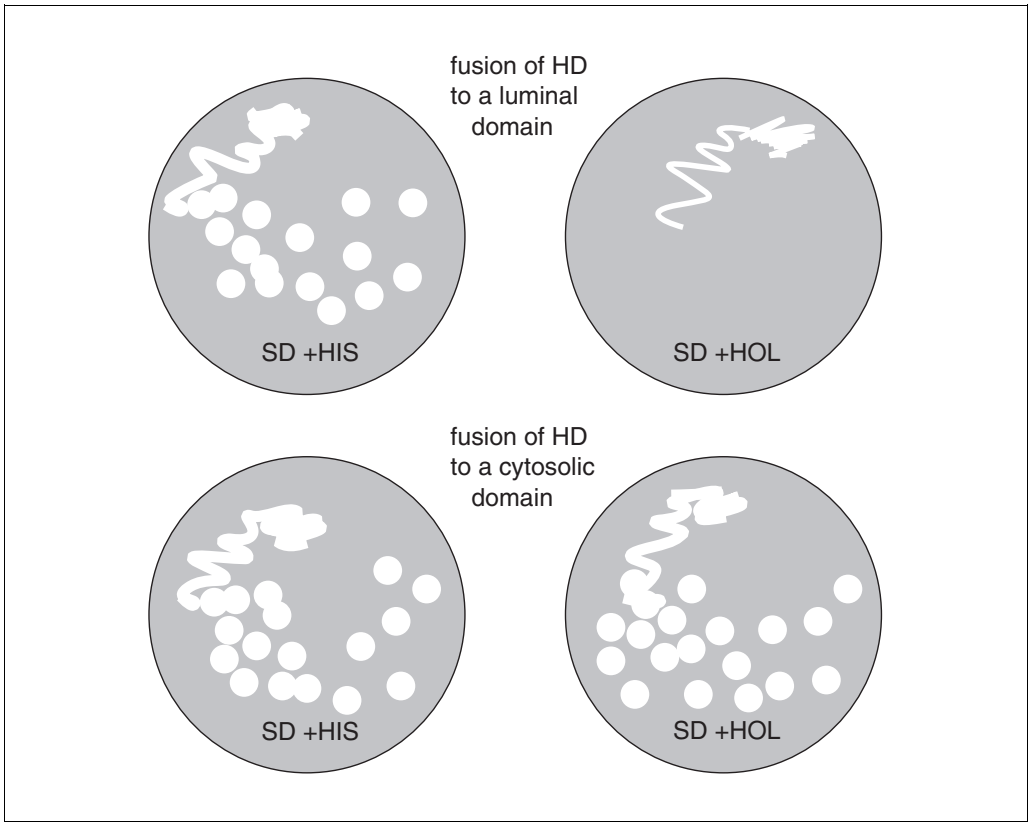


**Figure 5.2.8** Cell growth assay for determining membrane protein topology. Cells of yeast strain FC2-12B bearing a fusion protein that fuses the HD moiety to a luminal domain of an integral membrane protein or bearing a fusion protein that fuses the HD moiety to a cytosolic domain of an integral membrane protein are streaked for single colonies on SD +HIS agar plates and SD +HOL agar plates, then incubated 4 to 5 days at 30°C. When large colonies appear on SD +HIS agar plates and little growth is detected on SD +HOL agar plates, the reporter is interpreted to be fused to a luminal domain of the membrane protein under study. On the other hand, when large colonies appear on both the SD +HIS and SD +HOL agar plates, the reporter is interpreted to be fused to a cytosolic domain.

5. Transform a standard Amp$^s$ *E. coli* strain and isolate the desired plasmid construct.

   *A variety of E. coli strains can be used, such as strains HB101 and MC1061.*

***Perform genetic assay***

6. Transform the construct isolated from *E. coli* into *S. cerevisiae* strain FC2-12B and select transformants on SD +HIS agar plates.

   *Yeast cells should be handled using aseptic technique.*

   *Strain FC2-12B contains a ura3 mutation that permits selection for pA189invHD-derived plasmids. Transformants should appear as individual colonies after 4 days on agar plates incubated at 30°C.*

7. Test transformed yeast cells for growth by streaking cells on SD +HOL agar plates and SD +HIS agar plates.

   *Individual colonies should appear after 4 to 5 days at 30°C. An example of the results expected is shown in Figure 5.2.8. As shown in this figure, fusion of HD to a luminal or cytosolic domain does not affect cell growth on SD +HIS agar plates. However, fusion of HD to a luminal domain inhibits growth on SD +HOL agar plates. Fusion of HD to a cytosolic domain does not inhibit cell growth on either type of agar plate.*

## REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see* ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

***DMEM/FBS/HEPES (DFH) solution***

10% (v/v) fetal bovine serum
20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4
Prepare in supplemented Dulbecco's modified Eagle medium (DMEM; *APPENDIX 2A*)
Prepare fresh

*The fetal bovine serum should not be heat-inactivated.*

***Magnesium/sucrose/BSA (MSB) buffer***

150 mM potassium acetate
5 mM magnesium acetate
50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.6
200 mM sucrose
1 mM dithiothreitol (*APPENDIX 2A*)
Prepare fresh

***Nonidet P-40/goat serum/BSA (NGB) solution***

0.05% (v/v) Nonidet P-40 (Igepal AC-630)
5% (v/v) goat serum
1% (w/v) BSA
Prepare in PBS (*APPENDIX 2A*), pH 7.4
Prepare fresh

***Paraformaldehyde, 4%***

Heat 900 ml water to 55° to 60°C on a stirring hot plate in a fume hood. Add 40 g paraformaldehyde powder and stir 30 min. If powder has not dissolved, add a few NaOH pellets one at a time (waiting a few minutes between pellets) until the paraformaldehyde dissolves. Add 100 ml of 10× PBS (*APPENDIX 2A*), filter, cool to room temperature, and adjust to pH 7.4 with HCl. Store up to 1 week at 4°C.

CAUTION: *The fume hood is used because paraformaldehyde fumes are toxic. The "Prill" form of paraformaldehyde from EM Sciences is safest to use, as it pours without creating a cloud.*

***Proteinase K, 10 mg/ml***

10 mg proteinase K (lyophilized powder, ~80% protein, 10 to 20 U/mg protein; Sigma)
1 ml 50 mM HEPES, pH 7.6
Prepare fresh on ice

*No activation of the proteinase K is necessary; the purchased enzyme is ready to use.*

***SD +HIS agar plates***

0.7 g/liter yeast extract (without amino acids; Difco)
20 g/liter glucose
20 g/liter agar
0.1 mg/ml L-tryptophan
0.1 mg/ml L-leucine
0.1 mg/ml L-histidine

Mix the ingredients together before autoclaving. Pour the autoclaved solution into 100 × 15–mm petri dishes. Store plates <1 month at 4°C.

***SD +HOL agar plates***

0.7 g/liter yeast extract (without amino acids; Difco)
20 g/liter glucose
20 g/liter agar
0.1 mg/ml L-tryptophan
0.1 mg/ml L-leucine
0.1 mg/ml L-histidinol dihydrochloride (Sigma)

Filter-sterilize the histidinol solution and add to the remainder after autoclaving. Store plates <1 month at 4°C.

***TCA, 100%***

250 g solid trichloroacetic acid (TCA)
141 ml water
Store <6 months at 4°C

## COMMENTARY

### Background Information

Integral membrane proteins can often be distinguished from peripherally bound proteins in that the latter are extractable from the membrane under conditions of high pH (Steck and Yu, 1973; Fujiki et al., 1982; Russel and Model, 1982). Resistance of integral membrane proteins to extraction is attributed to the presence of transmembrane segments which help anchor proteins to the lipid bilayer. Visual and computer-based inspections of the primary sequences of integral membrane proteins usually reveal one or more hydrophobic amino acid stretches interspersed among hydrophilic stretches in an alternating pattern. This pattern suggests a topology in which neighboring hydrophilic domains are positioned on opposite sides of a membrane through integration of hydrophobic transmembrane regions. These topological predictions have been generally confirmed through an emerging number of crystal structures of integral membrane proteins (Deisenhofer et al., 1985; Xia et al., 1997). Despite the availability of methods for X-ray diffraction analyses of membrane proteins, topological studies still occupy an important place in protein structural studies. This is due to the fact that structures of membrane proteins are much less easily obtained than those of water-soluble proteins because of difficulties in obtaining crystals. As most integral membrane proteins require a specific topology for function, determining the orientations of hydrophilic domains may provide significant insight into the proteins' roles in vivo.

#### *Protease digestion*

Protease digestion is one of the oldest methods for determining membrane protein topology. This approach relies on the principle that the lipid bilayer is generally impermeable to proteases. Therefore, addition of a protease to the outside surface of a membrane vesicle results in degradation of protein sequences lo-

cated on the exterior surface, thereby revealing the luminal content (Gerber et al., 1977). It is best to examine a protein in its native membrane environment. The protease digestion protocol described in this unit (see Basic Protocol 1) is useful for examining proteins residing within isolated ER membrane vesicles by immunoblotting, although this procedure can be adapted easily to the study of proteins present in the plasma membrane and in other membrane-bound organelles. The primary difference is in the enrichment procedure used to isolate the desired membrane preparation (Li and Shore, 1992; Graham et al., 1994; Kalish et al., 1995).

Alternatively, one may examine the topologies of proteins integrated into microsomal vesicles in vitro. Such proteins are found to assume a specific topology that can be probed by the addition of a protease (Mize et al., 1986). In vitro–expressed proteins can, however, suffer the problem of being integrated inefficiently (Skach et al., 1994), and in vitro systems may not offer a functional assay assuring that the membrane protein has achieved its native structure. However, the membrane integration machinery in the ER is believed to perform accurately in vitro, at least with native membrane proteins (see *UNIT 11.4*).

In order to gain a complete picture of a protein's topology using the protease digestion method, each hydrophilic domain must be inherently sensitive to the protease used. That is, accurate pictures are obtained only when each domain is digestible either at the surface of the vesicle or in detergent-solubilized solutions. To ensure that the hydrophilic domain being probed can be proteolytically cleaved, it should contain a site recognized by the protease used. Proteases exhibiting a broad substrate specificity, such as proteinase K and trypsin, should therefore be used.

For particularly small domains, accessibility to a protease may be a problem. Furthermore, since digested regions are probed with antibodies directed against a series of peptides corresponding to various hydrophilic regions within the membrane protein, there is no guarantee that all of the antibody preparations will recognize the intact protein (Seckler et al., 1986). Despite these potential problems, the protease digestion approach is a proven method, particularly as it has been used to probe large hydrophilic domains.

***Immunofluorescence staining***

Immunofluorescence staining has been used widely to analyze the topology of proteins localized to the plasma membrane (Canfield and Levenson, 1993; Canfield et al., 1996). This method (see Basic Protocol 2) has the advantage that domains present on either side of the membrane can be visualized with a microscope, as opposed to detection of proteolytic fragments which may be small and difficult to find by SDS-PAGE. Compared to the protease digestion approach, immunofluorescence staining has not been used generally to study membrane protein topology in organelle-based systems. It should be possible, however, to identify hydrophilic domains located at the surface of peroxisomes in mammalian cells by taking advantage of the fact that the plasma membrane, but not the peroxisomal membrane, is permeabilized with the detergent digitonin (Swinkels et al., 1991). Since both plasma and peroxisomal membranes are permeabilized after addition of Triton X-100, use of these detergent combinations should permit a topological analysis of peroxisomal proteins.

***Epitope tagging***

Preparation of antipeptide antibodies needed for Basic Protocols 1 and 2 is costly and time-consuming. These problems are exacerbated by the fact that many antipeptide antibodies fail to recognize their corresponding epitope within the intact membrane protein. An alternative approach, epitope tagging, is therefore presented in this unit (see Support Protocol). A DNA fragment encoding a foreign epitope recognized by a commercially available antibody is introduced into the gene encoding a membrane protein. A tagged domain can then be identified using protease digestion (see Basic Protocol 1) or immunofluorescence staining (see Basic Protocol 2; Canfield and Levenson, 1993; Skach et al., 1994; Canfield et al., 1996).

There are, however, disadvantages that may result from the addition of an epitope. The epitope may render the membrane protein into which it has been inserted nonfunctional (Canfield et al., 1996). In such cases, it is not always clear whether the topology has been disrupted or whether the epitope has been inserted into a site critical for activity. In either event, results derived from studies of a nonfunctional protein are unreliable in the absence of supportive topological data. Even if the tagged protein is functional, the possibility remains that the foreign

epitope has disrupted the topology of a region unimportant to the protein's activity under the conditions measured.

Although the HA epitope has been used successfully for determining membrane protein topology (Canfield and Levenson, 1993), it contains two charged amino acids (YPYDVPDYA; Wilson et al., 1984). A number of studies have shown that charged residues located near membrane-spanning sequences play a role in determining transmembrane orientation in eukaryotic cells. Indeed, introduction of clusters of negatively charged amino acids proximal to transmembrane segments can inhibit their integration (Green and Walter, 1992). However, the HA epitope contains fewer charged amino acids than FLAG (DYKDDDDK; Hopp et al., 1988), c-myc (EQKLISEEDL; Evan et al., 1985), and VSV-G (YTDIEMNRLGK; Kreis, 1986) epitopes and is therefore recommended for use. The AU1 epitope (DTYRYI) derived from the papillomavirus major capsid protein also contains only two charged amino acids (Lim et al., 1990); however, it has not yet been utilized for topological analysis to the extent that the HA epitope has.

To avoid interfering further with integration of the target protein, the foreign epitope should be placed ≥15 amino acids from the neighboring transmembrane segment(s) without disrupting membrane targeting signals which are commonly present at the N- and/or C-terminal ends of the polypeptide chain. Another concern is that addition of artificial sequences into a membrane protein may affect its stability or localization to a compartment within the cell. Indeed, retention of mutant proteins in the secretory pathway is well documented (Gething et al., 1986; Kreis and Lodish, 1986). Loss of stability or missorting may be evidenced through failure to find the tagged protein in its normal cellular location.

### *Reporter gene fusions*

Since the mid-1980s, a genetic approach has been used to solve membrane protein topologies (see Basic Protocol 3). The reporter gene fusion method was developed originally to study the topology of plasma membrane proteins in bacterial systems (Manoil and Beckwith, 1986; Froshauer et al., 1988) and then adapted for the study of proteins integrated into the ER membrane in the yeast *Saccharomyces cerevisiae* (Sengstag et al., 1990). More recently, the reporter enzyme β-galactosidase has been used for topological studies in *Caenorhabditis elegans* (Doan et al., 1996; Lai et al., 1996). Reporter fusion approaches developed for analyses in yeast and *C. elegans* examine membrane proteins integrated into the ER and either residing within the ER or transported to a compartment of the secretory pathway or to the lysosome. Due to conservation of the ER membrane integration machinery in diverse eukaryotic organisms (Hartmann et al., 1994), cross-species analyses of eukaryotic proteins using the simple yeast system are probably valid. Recently, a gene fusion system has been used to study the topology of a protein localized to the peroxisomal membrane (Elgersma et al., 1997), and it is likely that over the next few years the reporter gene fusion method will be extended to other organelles and eukaryotic cell types.

The cytoplasmic enzyme histidinol dehydrogenase (HD) is well suited for use as a reporter of membrane protein topology in yeast cells (Sengstag et al., 1990). HD is encoded by the *HIS4* gene in *Saccharomyces cerevisiae* (Donahue et al., 1982). When expressed on the cytosolic side of the membrane, HD converts histidinol to histidine, an activity that permits yeast that are *his4*$^{-}$ to grow on agar plates supplemented with histidinol instead of histidine. On the extracytoplasmic side, however, HD is enzymatically inactive in vivo, presumably due to its sequestration from the histidinol substrate, glycosylation of the HD moiety, and/or degradation of HD within the ER lumen (Green and Walter, 1992; Mullins et al., 1995).

To construct gene fusions in pA189invHD, the 800-bp *Bam*HI-*Xho*I fragment can be replaced with a DNA fragment encoding an N-terminal truncation of the target membrane protein. This leads to the production of fusions between the upstream membrane protein fragment and the C-terminal inv-HD sequence. In pA189invHD, the inv sequence (an internal fragment of the yeast invertase protein; Taussig and Carlson, 1983) serves as a spacer that separates HD from the membrane-spanning segments of the upstream membrane protein (Green and Walter, 1992).

The main advantage of the reporter gene fusion approach described here is its simplicity. The cell growth assay provides a quick and reliable means of assessing the location of a hydrophilic domain within a membrane protein examined in yeast. Furthermore, a fusion joint can be constructed anywhere along the primary sequence using standard molecular biological protocols. This means that all hydrophilic domains within a multispanning protein, includ-

ing small domains, can, in principle, be examined by constructing a series of gene fusions. However, such fusions eliminate from the construct all sequences in the membrane protein located downstream of the fusion joint. This approach therefore makes the assumption that membrane proteins integrate into the bilayer in a linear fashion, from the N to the C terminus. This idea is in accord with the model that membrane proteins contain alternating signal and stop transfer signals positioned linearly along the primary sequence (Friedlander and Blobel, 1985). While this assumption may be accurate in some instances, the fusion approach cannot identify transmembrane domains that insert into the membrane with the help of C-terminal sequences (Finer-Moore and Stroud, 1984).

In light of current models suggesting that multiple transmembrane domains accumulate within the translocation channel before their entry into the lipid bilayer (Hegde and Lingappa, 1997), caution should be used when interpreting results obtained using only the reporter fusion approach. For this reason, combining the gene fusion approach with one of the other approaches described in this unit will not only yield a more reliable topological picture but may also provide information about the mechanism of integration of the protein examined.

## Critical Parameters

### *Protease digestion*

Several factors are important for interpreting membrane protein topology using the protease digestion method. Determining the location of a hydrophilic domain that is at the side of the membrane to which the protease has not been added can be difficult, if the domain in question is small. This is due to the fact that digestion of those portions exposed to the protease may leave a fragment too small to detect using SDS-PAGE. Thus, the failure to detect a protein fragment may be due to a detection problem, not to the susceptibility of the domain to the protease added (see Anticipated Results). On the other hand, a domain exposed to the protease may be resistant or inaccessible if the domain is small. Therefore, it is critical to interpret carefully results regarding the disposition of a small domain. To assign a small hydrophilic domain to the correct side of the membrane, it is best to examine the topology over the entire protein, then determine whether the results obtained are consistent with the deduced assignment of the domain(s) in question. One way to enhance the chance that a small domain is susceptible is to use a protease that has a broad substrate specificity, such as proteinase K or trypsin. The use of a control protein or protein domain that faces the luminal side of the membrane system to be probed is essential to the analysis, since the membranes examined must be closed and impermeable to the protease added in the absence of detergent.

### *Immunofluorescence staining*

There are several technical features of the immunofluorescence staining protocol that are critical to a successful outcome. The use of very clean coverslips is necessary. Coverslips should be washed in a strong detergent and rinsed thoroughly to remove all traces of the detergent. The adherence of tissue culture cells to the coverslips can be a problem sometimes. The protocol described here works well for HEK 293 cells, but when other cells are used, different types of coverslips can be tried. For example, Lab-Tek Chamber Slides (Nunc) provide a surface to which many cell types attach well. When using HEK 293 cells, it is observed that cells which are about 70% confluent yield the best staining results. Using the procedures outlined here, it takes about 3 days following seeding on coverslips to achieve this confluency; however, different times may be necessary in different experiments. In addition, these conditions may need to be varied for cell types other than HEK 293 cells.

The most important control is to examine cells lacking the protein of interest. This control detects the background staining that may appear in the cells under study. If the cells containing the protein of interest give a much more intense staining than cells lacking the protein, then the results should be reasonably reliable. The protocol listed here calls for a membrane protein control to be analyzed. This control is very useful, provided that antibodies directed against this control protein are available. If not, then it is essential that at least one hydrophilic domain located on the intracytoplasmic side of the membrane be probed, as such a domain controls for the possible disruption of the membrane prior to addition of the primary antibody.

### *Epitope tagging*

Epitope tagging offers an inexpensive shortcut to analyzing membrane protein topology using either the protease digestion or the immunofluorescence staining protocol. However, there is one issue that is important in achieving the reliability necessary. The results are more

certain to be valid if the epitope does not inhibit function of the target protein. It is therefore important to examine the function of the epitope-tagged protein using an in vivo or in vitro assay. To improve the chance that the epitope does not alter the topology of a protein (and thus its function), an epitope lacking clusters of charged amino acids, such as the HA epitope, should be used. Furthermore, the epitope should be placed >15 amino acids away from the upstream and/or downstream transmembrane segments, in order to avoid disrupting the content of charged amino acids already existing near the transmembrane segments. If it is not possible to obey this 15 amino acid restriction (because the domain probed is small) then place the epitope as far away as possible from the transmembrane segment(s).

***Reporter gene fusions***

When membrane protein topology is assessed using an in vivo assay (such as the one described here), the reporter gene fusion approach is the quickest way to determine membrane protein topology. However, the fusions constructed usually render the target protein nonfunctional. Topological assignments are therefore best served by using this method to gain only a general picture of a protein's topology and then complementing the data with at least a partial analysis using one of the above-listed approaches. Since a functional assay is not always practical, there are some issues to consider when constructing the fusions in order to minimize the chance that the reporter affects the topology of the protein being probed. The fusion joint should be constructed >15 amino acids away from the upstream transmembrane segment. For small hydrophilic domains, the fusion joint can be placed after the first few amino acids of the downstream transmembrane segment if one exists (see Fig. 5.2.6). Another factor that is important is the amino-terminal sequence of the reporter itself. When the reporter contains clusters of charged amino acids at its amino terminus, the reporter may inhibit the integration of the upstream transmembrane segments. Indeed, the His4p reporter appears to suffer this problem (Green and Walter, 1992). For this reason, the reporter construct described in this protocol contains a spacer sequence consisting of a fragment of invertase (Fig. 5.2.7).

## Troubleshooting

Refer to Table 5.2.1 for a troubleshooting guide.

## Anticipated Results

A typical result expected from the protease digestion method (see Basic Protocol 1) is shown in Figure 5.2.2A. Note that no apparent fragment is detected from proteolysis of the representative protein SPC12 in the absence of detergent (lane 2). If a fragment had been detected, the result would have suggested that the C terminus localizes to the luminal side of the membrane. However, such a fragment could have been quite small (as few as 20 to 30 amino acids) if the C-terminal hydrophilic domain contained only a few amino acids. A clue as to the expected size of a particular fragment can be obtained by examining a hydropathy plot of the protein. Data similar to those shown in Figure 5.2.2A are expected from the analysis of epitope-tagged proteins (see Support Protocol), except that instead of antipeptide antibodies, antibodies directed against the epitope employed should be used. Results expected from immunofluorescence analyses (see Basic Protocol 2) of epitope-tagged proteins have been reported (Canfield and Levenson, 1993; Canfield et al., 1996). Results expected from reporter fusion analyses (see Basic Protocol 3) in yeast (Sengstag et al., 1990) and *C. elegans* (Doan et al., 1996; Lai et al., 1996) have also been reported.

## Time Considerations

The time frames given below are for procedures that work well in the first few attempts. Longer times will, of course, be needed when significant troubleshooting is required.

***Protease digestion***

Antipeptide antibodies directed against each hydrophilic domain of the target protein can take 3 months or longer to prepare. This time can be spent working out the conditions for preparing enriched membranes containing the protein of interest. If control antibodies are available, they can be used to verify the integrity of the membranes being prepared. It usually takes no more than 2 to 3 days to obtain most membrane preparations. The protease treatments and SDS-PAGE analyses also take 2 to 3 days (see Basic Protocol 1).

***Immunofluorescence staining***

As with the protease digestion technique, most of the time is spent preparing a series of antipeptide antibodies. This time is lessened if commercially available epitope tags are used (see Support Protocol). When examining cells grown in tissue culture medium (see Basic

**Table 5.2.1** Troubleshooting Guide for Problems Encountered in Determining Membrane Protein Topology

| Problem | Possible cause | Solution |
|---|---|---|
| ***Protease digestion*** | | |
| Target protein is not degraded in detergent. | Protein is resistant to protease used. | Try another protease. |
| | Protein is shielded by other proteins. | Treat membrane with buffer containing 500 mM potassium acetate and add 500 mM potassium acetate to the detergent control. To release ribosomes from ER microsomes, treat with MSB containing 500 mM potassium acetate, 0.2 mM GTP, 1 mM puromycin. |
| | Protein is part of a protease-resistant complex. | Preincubate membranes 10 min at 40°-60°C. Monitor membrane integrity using a control membrane protein. |
| The target protein is digested in the detergent but not in intact vesicles. | The protein may lack a cytoplasmic domain or is shielded. | Treat membranes with 500 mM potassium acetate as described above before adding the protease. |
| Poor proteolysis of target and control proteins is found. | Protease inhibitors or nonfunctional protease is present. | Ensure inhibitors are absent from membrane preparation by washing membranes by high-speed centrifugation (see Basic Protocol 1, step 1). Be sure protease is active by examining proteolyzed and unproteolyzed membranes using SDS-PAGE (*UNIT 6.1*). |
| Degradation of a luminal protein control occurs even without addition of detergent. | Too much protease is used. | Titrate protease to lower concentrations than those indicated in Basic Protocol 1. |
| | The membrane is leaky. | Change the cell fractionation protocol (Chapter 3). |
| ***Immunofluorescence staining*** | | |
| The antibody used does not recognize the target protein in the permeabilized and nonpermeabilized sets. | The antibody used binds the target protein inefficiently in the context of the membrane. | Test whether antibody binds the protein by immunoblotting (*UNIT 6.2*) or immunoprecipitation (*UNIT 7.2*) from detergent-solubilized membranes. |
| | | If the hydrophilic domain being probed is small, try inserting one or more epitopes into the domain (see Support Protocol) and repeat analysis. |
| Hydrophilic domains presumed to be located on opposite sides of the membrane are detected in both the permeabilized and nonpermeabilized sets. | The nonpermeabilized cells were leaky at the time primary antibody was added. | Be sure that detergent was not added to DFH solution.<br>Examine a control protein with known topology. |
| Control cells lacking the polypeptide of interest are stained strongly by the secondary antibody. | The concentrations of primary and/or secondary antibodies are tood high. | Test whether dilution of the antibodies decreases the background signal.<br>Try using a different primary antibody preparation. |
| ***Epitope tagging*** | | |
| The tagged protein is not functional. | The protein is degraded, mislocalized, or out of frame. | Test whether protein is present in its native membrane by immunoblotting (*UNIT 6.2*) or immunoprecipitation (*UNIT 7.2*). Sequence the gene segment encoding the epitope and flanking amino acids. |

*continued*

**Table 5.2.1** Troubleshooting Guide for Problems Encountered in Determining Membrane Protein Topology, continued

| Problem | Possible cause | Solution |
|---|---|---|
| | The epitope inhibits the active site or topology of the protein. | Place the epitope at a different site in the protein domain, including sites further away from the transmembrane stretches. Alternatively, proceed with topology assays, but corroborate results using a different method. |
| The antibody does not recognize an epitope-tagged protein even though the protein is functional. | The epitope is inaccessible. | Introduce multiple epitopes into the site. |
| ***Reporter gene fusions*** | | |
| Fusions to hydrophilic domains on both sides of a putative transmembrane segment possess HD enzymatic activity. | The L-histidinol reagent is contaminated with histidine. | Test whether strain FC2-12B grows on SC +HOL agar plates. If it does, try a different batch of histidinol or a different supplier. |
| | The hydrophobic segment identified does not actually span the membrane, or the transmembrane segment inserts into the bilayer aided by downstream sequences not contained in the fusion protein. | Reassess whether the hydrophobic segment is a transmembrane segment. Monitor topology of the region in question using a different approach. |
| None of the fusions possess enzymatic activity. | The fusions are not made. | Sequence fusion joints to ensure reading frame is maintained. Ensure that no ATG codons are present between the promoter and the initiation codon. Use the strong *ADH1* promoter on pA189invHD. |
| Fusions expected to be inactive are active. | The fusion joint interferes with integration of the upstream sequences. | Position the joint further away from the upstream transmembrane segment. Corroborate results using a different method. |

Protocol 2), it takes about 3 days to grow cells on coverslips before the immunofluorescence analysis. The analysis takes about 1 day, which includes fixing the cells, incubating with primary and secondary antibodies, and examining the cells by microscopy.

***Epitope tagging***

The time required to perform the molecular biological manipulations required to introduce a foreign epitope into each hydrophilic domain of a membrane protein (see Support Protocol) depends on the number of distinct hydrophilic domains present in the protein and whether single, double, or multiple tags are introduced into each domain. A person skilled in the use of molecular biological techniques could introduce a set of single and double tags into a type I integral membrane protein (Fig. 5.1.2) in <1 month, test the function of the various tagged proteins in vivo or in vitro in a few weeks, and localize the epitopes with respect to the membrane using protease digestion (see Basic Protocol 1) or immunofluorescence staining (see Basic Protocol 2) in ~1 week using either technique. Thus, in ~2 months, one could tag such a protein and examine its topology. Longer periods (up to 6 months) may be required to examine proteins containing several transmembrane segments.

***Reporter gene fusions***

Using the described systematic approach for constructing gene fusions (see Basic Protocol 3), a series of constructs can be made in <1 month by a person experienced in using molecular biological techniques. Analyses of the fusions require ~1 week using the yeast system or a few weeks using a higher eukaryotic system.

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