The Internal Repeats in the Na\textsuperscript{+}/Ca\textsuperscript{2\+} Exchanger-related *Escherichia coli* Protein YrbG Have Opposite Membrane Topologies*

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We have determined the topology of the *Escherichia coli* inner membrane protein YrbG, a putative Na\textsuperscript{+}/Ca\textsuperscript{2\+} exchanger with homology to a family of eukaryotic ion exchangers. Our results show that the two homologous halves of YrbG both have five transmembrane segments but opposite membrane orientations. This has implications for our understanding of the function of Na\textsuperscript{+}/Ca\textsuperscript{2\+} exchangers and provides an example of “divergent” evolution of membrane protein topology.

The *Escherichia coli* YrbG protein belongs to a superfamily of membrane transporters that includes both prokaryotic and eukaryotic Na\textsuperscript{+}/Ca\textsuperscript{2\+} exchangers (1). All proteins in this family have an internal repeat in their membrane domain that presumably has arisen from a primordial gene duplication event. A strongly conserved and functionally important so-called α-motif that spans two of the predicted transmembrane helices is present in each copy of the internal repeat (1, 2). Attempts to determine the membrane topology of the mammalian Na\textsuperscript{+}/Ca\textsuperscript{2\+} exchanger have yielded conflicting results (3–5), and it is not clear whether the two conserved α-motifs are located on the same or opposite sides of the membrane. Obviously, a resolution of this point will have important functional implications for this class of proteins.

In general, both prediction and experimental determination of membrane protein topology is easier for bacterial than for mammalian proteins, and we have thus undertaken a study of the *E. coli* family member YrbG. The sequence alignment in Fig. 1A shows clear evidence of the internal repeat, including the two α-motifs. Overall, there is ~34% identity between the N- and C-terminal halves of YrbG, and a BLASTP (6) search of the Entrez protein sequence data bank using the N-terminal half (residues 1–155) finds the C-terminal half (residues 172–320) with an E-value of 2 × 10\textsuperscript{-16} (data not shown).

Interestingly, topology predictions using TOPPRED II (7), TMHMM (8), and HMMPRED (9) suggest a topology with 10 transmembrane segments for YrbG, with the first and second internal repeat having opposite orientations in the inner membrane; see Fig. 1B. This is reminiscent of a recently described case of “divergent” topology evolution where two homologous *E. coli* inner membrane proteins, YdgQ and ORF193, each with 6 transmembrane segments, were shown to have opposite membrane orientations (10). The predicted topology for YrbG likewise suggests that a primordial protein with 5 transmembrane segments has evolved, after an internal gene duplication, into a protein with 10 transmembrane segments where the first and second halves insert into the membrane with opposite orientations. From this perspective, it is particularly noteworthy that the distribution of positively charged amino acids is different between the two halves of YrbG; in the N-terminal half, Arg and Lys residues are predominantly found in the loops between transmembrane segments 1/2 and 3/4, whereas in the C-terminal half they are more prevalent in the loop preceding the first transmembrane segment and between transmembrane segments 2/3 and 4/5; see Fig. 1B. The YrbG protein may thus be a second example of divergent topology evolution, this time following an internal gene duplication event.

Given the biological importance of the Na\textsuperscript{+}/Ca\textsuperscript{2\+} exchanger family and the topology evolution issue, we decided to experimentally map the topology of YrbG using the PhoA fusion approach (11). Our results provide strong support for the predicted topology with 5–5 transmembrane segments, placing the two α-domains on opposite sides of the membrane and suggesting that divergent topology evolution may be more prevalent than hitherto thought.

MATERIALS AND METHODS

Enzymes and Chemicals—Unless otherwise stated, all enzymes were from Promega (Madison, WI). T7 DNA polymerase, Taq polymerase, and [\textsuperscript{35}S]Met were from Amersham Pharmacia Biotech. T4 ligase was from Life Technologies, Inc. Oligonucleotides were from CyberGene (Stockholm, Sweden). PhoA antisera was from 5 Prime — 3 Prime, Inc. (Boulder, CO). The alkaline phosphatase chromogenic substrate PNPP (Sigma 104 phosphatase substrate) was from Sigma.

Strains and Plasmids—Experiments were performed in *E. coli* strains MC1061 (ΔλacX74, araD139, Δara, leu7697, galU, galK, hsr, hsm, strA) (12) and CC118 (Δara-leu7697, ΔλacX74, ΔphoA20, galE, gaiK, thi, rpsE, rpoB, argE, fam), recA1) (13). All constructs were expressed from the pING1 plasmid (14) by induction with arabinose.

DNA Techniques—All plasmid constructs were confirmed by DNA sequencing using T7 DNA polymerase. The yrbG gene was amplified by PCR from *E. coli* JM109 chromosomal DNA using Taq polymerase and the Expand Long Template PCR system (Roche Molecular Biochemicals). By use of appropriate PCR primers, a 5' *XhoI* and a 3' *KpnI* site were introduced in the regions flanking the amplified gene. The PCR products were cleaved with *XhoI* and *KpnI* and cloned behind the ara promoter in a *XhoI* site in codon 78. Relevant parts of the yrbG gene were amplified by PCR from the pLING1 plasmid with a 5' *SalI* and a 3' *KpnI* site encoded in the primers. Finally, the amplified *SalI*-[*KpnI* fragment carrying the *SalI* upstream region and the relevant *yrbG* segment was cloned into a previously constructed plasmid (15) carrying a *phoA* gene lacking the 5' *SalI* segment coding for the signal sequence and the first 5 residues of the mature protein and immediately preceded by a *KpnI* site. In all constructs, an 18-amino acid linker (YPYDSTYQVASWTEFP-FPC) was present between the *yrbG* part and the *phoA* moiety.

Expression of Fusion Proteins—*E. coli* strain CC118 transformed with the pLING1 vector carrying the relevant construct under control of

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[1] The abbreviation used is: PCR, polymerase chain reaction.
YrbG Topology

Fig. 1. A, LFASTA alignment of the N- and C-terminal halves of YrbG. The predicted transmembrane segments are underlined, and the conserved α-motifs are boxed. Note that each α-motif consists of two parts separated by a short, less well conserved loop. B, proposed topology for YrbG. The two homologous halves are indicated by black and hatched transmembrane segments, respectively, and the two α-motifs are shown by thick lines. The number of Lys + Arg residues is indicated in each loop. Each fusion is indicated by its number; the exact position of each fusion joint is given in the legend to Fig. 2.

Fig. 2. A, expression of YrbG-PhoA fusion proteins in E. coli CC118 cells. Cells were labeled for 2 min by [35S]Met, and fusion proteins were immunoprecipitated with a PhoA antiserum and analyzed by SDS polyacrylamide gel electrophoresis. Lane 1, YrbG77-PhoA; lane 2, YrbG272-PhoA; lane 3, YrbG209-PhoA; lane 4, YrbG128-PhoA; lane 5, YrbG325-PhoA; lane 6, YrbG275-PhoA; lane 7, YrbG209-PhoA; lane 8, YrbG325-PhoA; lane 9, YrbG209-PhoA; lane 10, YrbG275-PhoA. B, alkaline phosphatase activities for the different YrbG-PhoA fusions. Black bars indicate the measured activities (in Miller units) before correcting for expression levels, and white bars indicate activities after this correction (with the expression level for fusion 1 set to 1; see "Materials and Methods").

RESULTS

Determination of the Topology of YrbG by PhoA Fusions—To determine the membrane topology of YrbG, a series of PhoA fusions were made. As recommended (18), the fusion joints were generally placed near the C-terminal end of the putative periplasmic and cytoplasmic loops; see Fig. 1B. All fusions could be expressed in the phoA− strain CC118 (13), could be immunoprecipitated by a polyclonal PhoA antiserum, and were of the expected sizes; see Fig. 2A. Alkaline phosphatase activities were measured according to Ref. 16 and are shown in Fig. 2B.

The alkaline phosphatase activities are in general in very good agreement with the predicted topology. The only apparent exception is the somewhat high activity of fusion 1 placed in the loop between transmembrane segments 1 and 2. PhoA fusions directly after the first transmembrane segment in polypeptide proteins with an N terminus (i.e., with a periplasmic N terminus) often give high activities, and there are now a number of examples where it has been found that more than one transmembrane segment needs to be present to ensure translocation of an N-terminal tail across the membrane (19). Because the hydrophobicity profile is very distinct in this area of the protein, and because all the other fusions are consistent with the predicted overall Nout-Cout topology, it seems likely that the high "uncorrected" activity of the most N-terminal segment was underpredicted.

## Topology Prediction and Sequence Alignment—

Topology predictions were done using TOPPRED II (7), TMHMM (8), and HMMTOP (9). Sequence alignments were done using BLAST and PSI-BLAST (6) at the NCBI website and LFASTA (17) at the Biology Workbench 3.0 website on the Internet. Default parameter settings were used in all cases.

The arabinose promoter was grown at 37 °C in M9 minimal medium supplemented with 100 μg/ml ampicillin, 0.5% fructose, 100 μg/ml thiamin, and all amino acids (50 μg/ml each) except methionine. An overnight culture was diluted 1:25 in fresh medium, shaken for 3.5 h at 37 °C, induced with arabinose (0.2%) for 5 min, and labeled with [35S]methionine (75 Ci/ml). After 2 min, samples were acid-precipitated with trichloroacetic acid (10% final concentration), resuspended in 10 mM Tris/2% SDS, immunoprecipitated with antisera to PhoA, washed, and analyzed by SDS polyacrylamide gel electrophoresis. Gels were scanned in a FUJIX Bas 1000 phosphorimager and analyzed using the MacBAS software (version 2.31).

PhoA Activity Assay—Alkaline phosphatase activity was measured by growing strain CC118 transformed with the appropriate pING1-derived plasmid in liquid culture for 2 h in the absence of arabinose and then for 1 h in the presence of 0.2% arabinose (16). Mean activity values were obtained from three independent measurements and were normalized by the rate of synthesis (mean of three experiments) of the protein, and because all the other fusions are consistent with the predicted overall Nout-Cout topology, it seems likely that the high "uncorrected" activity of the most N-terminal segment was underpredicted.

**Equation 1**

\[ A = \frac{A_0 \times OD_{600 \times N_{\text{Met}} \times CPM}}{n_{\text{Met}} \times N_{\text{cpm}}} \] (Eq. 1)

where \( A_0 \) is the measured activity, OD_{600} is the cell density at the time of pulse labeling, \( n_{\text{Met}} \) is the number of Met residues in the fusion protein, and CPM is the intensity of the relevant band measured on the phosphorimager.
PhoA fusion does not properly reflect the orientation of the first transmembrane segment in the full-length protein. We conclude that YrbG has a two-domain structure where each domain has five transmembrane segments but where the two domains have opposite orientations in the inner membrane. This also places the two conserved α-motifs on opposite sides of the membrane. The 5+5 topology implies that YrbG has undergone a process of “internal” divergent topology evolution after the primordial gene duplication event.

**DISCUSSION**

In this study, we have mapped the topology of the *E. coli* inner membrane protein YrbG, which belongs to a large family of Na\(^+\)/Ca\(^2+\) exchangers that includes both prokaryotic and eukaryotic proteins. Sequence alignment, topology prediction, and experimental topology mapping using PhoA fusions strongly suggest a two-domain structure, each with five transmembrane segments and with opposite orientations in the membrane. As shown in Fig. 1B, this places the two copies of the conserved α-motif on opposite sides of the membrane, suggesting that this will also be the case for the eukaryotic family members. Some experimental support for this idea has been obtained previously (5). In terms of three-dimensional structure, this means that proteins in the Na\(^+\)/Ca\(^2+\) exchanger family will have a quasi-symmetric organization relative to the plane of the membrane, somewhat reminiscent of the aquaporins where two copies of the so-called NPA loop, one entering the membrane from the cytoplasmic side and one from the extracytoplasmic side, are thought to interact in the middle of the pore (20).

Equally interesting, YrbG provides a clear example of divergent topology evolution where the two homologous halves of the protein have evolved opposite orientations in the membrane. Presumably, the redistribution of positively charged Arg and Lys residues between the different loops in the protein underlies this evolutionary process, as has been suggested earlier for the two homologous *E. coli* inner membrane proteins YdgQ and ORF193 (10). In fact, the bacterial homologues of YrbG found by a PSI-BLAST search of the Entrez protein sequence data base all have the same predicted N\(_{\text{out}}\)-C\(_{\text{out}}\) topology with 10 transmembrane segments, except for the *Synechocystis* homologue (srl0681), which has an extra predicted C-terminal transmembrane segment not present in the other proteins, and all have the same enrichment of positively charged residues in their predicted cytoplasmic loops as seen for YrbG (data not shown).

Whether the postulated primordial YrbG half-protein with five transmembrane segments had a single orientation in the membrane or could insert both ways (N\(_{\text{in}}\)-C\(_{\text{out}}\) and N\(_{\text{out}}\)-C\(_{\text{in}}\)) as is the case for, e.g., ducin (21), is unknown. If it had only a single orientation, it is unlikely that a topology with 10 transmembrane segments would be adopted by anything but a very small fraction of the molecules immediately after the gene duplication. Instead, the majority would most likely adopt a so-called “leave one out” topology where one of the hydrophobic segments would not insert across the membrane, and all highly charged loops would remain on the cytoplasmic side (22). A considerable number of mutational events would then be required before most molecules would insert into the membrane with the N\(_{\text{out}}\)-C\(_{\text{out}}\) topology seen today. In any case, our results underscore the importance of the “positive inside” rule (23, 24) for determining membrane protein topology and suggest that divergent topology evolution or even topology inversion may be a more prevalent phenomenon than hitherto thought.

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