Topological analysis of the human $\beta_2$-adrenergic receptor expressed in Escherichia coli

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ABSTRACT We have investigated the topology of the human $\beta_2$-adrenergic receptor expressed in Escherichia coli, using the genetic method described by Beckwith and coworkers. We found that fusions with alkaline phosphatase beyond a certain point on the human $\beta_2$-adrenergic receptor sequence were assembled into the bacterial membrane with the same topology as the human $\beta_2$-adrenergic receptor in the mammalian membrane. The pattern that might have been expected on the basis of the topology of the human $\beta_2$-adrenergic receptor in mammalian membranes was not reflected in the levels of alkaline phosphatase activity of the fusions occurring between the N-terminal region and positions close to the second external domain. Our data suggest that the correct positioning of the N terminus of the receptor depends on the presence of its C-terminal portions.

The amino acid sequence of an integral membrane protein can be used to predict its membrane-spanning stretches. A plot of average hydrophobicity is used to draw plausible two-dimensional membrane topologies of the polypeptide in which each long hydrophobic sequence corresponds to a transmembrane $\alpha$-helix (1). X-ray diffraction analysis of bacteriorhodopsin and Rhodopseudomonas viridis photosynthetic reaction-center polypeptides has shown that, in fact, the long hydrophobic sequences correspond to transmembrane $\alpha$-helical stretches in these proteins (2). High-resolution structural analysis, however, has been possible for only a few membrane proteins, because of the difficulty of growing crystals suitable for diffraction.

Various alternatives to x-ray analysis have been developed to test models of protein topology with respect to a membrane. Beckwith and coworkers (3, 4) have described a genetic approach for analyzing the arrangement of integral membrane proteins in Escherichia coli. The catalytic domain of the bacterial alkaline phosphatase (PhoA), with its leader sequence missing, is fused to various portions of a membrane protein. PhoA acquires enzymatic activity only when it is translocated to the periplasm (5), where its intrachain disulfide bonds can form (6); its fusion to an integral membrane protein, therefore, will show different activities depending on where in the protein the PhoA fuses. Fusions to or near the periplasmic domains lead to higher PhoA activities than fusions to positions in the cytoplasmic domains. Using this method, Beckwith and others were able to determine and/or confirm the topological structure of several E. coli membrane proteins. The application of the method or of analogous ones (7) is limited to bacterial proteins or to proteins which are expressed and can fold correctly in a bacterial membrane.

Human $\beta_2$-adrenergic receptor (h$\beta_2$AR) is part of a family of membrane receptors which couple with guanine nucleotide-binding regulatory proteins (G proteins) (8). Hydrophobicity plots suggest that, like bacteriorhodopsin (9), these receptors are characterized by seven hydrophobic transmembrane segments, separated by extra- and intracellular hydrophilic loops. The topographical disposition of all eight of the hydrophilic sequences of h$\beta_2$AR expressed in mammalian cells was established with site-directed anti-peptide antibodies and in situ analysis by indirect immunofluorescence (10).

h$\beta_2$AR expressed in E. coli displays affinities for adrenergic ligands similar to those observed in eukaryotic cells (11-13) and is able to couple with G$_\alpha$, the $\alpha$ subunit of the G protein that stimulates adenylate cyclase. It appears, therefore, that the essential requirements for the accurate folding of this human receptor exist in E. coli.

We have analyzed the assembly of this eukaryotic membrane protein in E. coli by using a set of h$\beta_2$AR–PhoA fusions in order to determine the topology in the bacterial membrane and the requirements for the correct insertion in the membrane. Our data confirm the h$\beta_2$AR topology in a bacterial membrane predicted by hydropathy considerations and previously proven in the eukaryotic membrane. Moreover, the results of our analysis indicate that the correct positioning of the N terminus is dependent on the presence of C-terminal portions of h$\beta_2$AR.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Plasmids. The E. coli K-12 strain used was CC118-F' (3). The coding region of the h$\beta_2$AR, kindly provided by R. J. Lefkowitz (15), was subcloned in pUC18.

Plasmid pPH07 (16), a gift from J. Beckwith, is a derivative of pTZ with the PhoA coding sequence limited by different restriction sites. Bacteriophage $\lambda$:TnphoA, kindly provided by J. Beckwith, was used as a source of TnphoA (17).

Binding Assay of Recombinant h$\beta_2$AR. Ligand binding was assayed by a replica filter technique (12).

Construction of Fusions to Alkaline Phosphatase. phoA gene fusions were obtained in vivo by infecting strain CC118-F' with $\lambda$:TnphoA (17). In vitro fusions were obtained by using the plasmid pPH07 (16).

Assay of Alkaline Phosphatase. Exponentially growing cells were induced with isopropyl $\beta$-D-thiogalactopyranoside (IPTG, 20 $\mu$g/ml) for 2 hr and assayed as described (5).

Immunoprecipitation Conditions and Trypsin-Accessibility Experiments. To detect h$\beta_2$AR–PhoA hybrid proteins, exponentially growing cells (500 $\mu$l) in M9 medium/0.5% glycerol supplemented with 19 amino acids (minus methionine) were induced with IPTG, pulse labeled with 50 $\mu$Ci of $[^{35}$S]methionine (1 $\mu$Ci = 37 kBq) for 30 sec at 30°C, cooled in the presence of unlabeled methionine (100 $\mu$g/ml), and

Abbreviations: h$\beta_2$AR, human $\beta_2$-adrenergic receptor; $[^{125}$I]CYP, $[^{125}$I]iodocyanopindolol; IPTG, isopropyl $\beta$-D-thiogalactopyranoside.

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filters took place of induced with phatase or to albumin). Antibody ing to and buffer, Tris-HCl, mM incubated EDTA or phosphatase peroxidase-conjugated twice washed suspended conjugated anti-mouse 1.

Trypsin accessibility experiments were carried out according to Traxler et al. (19).

ELISA on Spheroplasts. Exponentially growing bacteria were induced with IPTG. Portions (0.5 ml) were pelleted and suspended in the same volume of 40% sucrose/35 mM Tris-HCl, pH 8.0. Spheroplasts were generated by adding EDTA (2.5 mM) and lysozyme (5 µg/ml) and incubating at 0°C for 15 min. Spheroplasts were then centrifuged at 4°C for 15 min and suspended in 0.5 ml of STEB (40% sucrose/35 mM Tris-HCl, pH 8.0/2.5 mM EDTA/0.1% bovine serum albumin). Antibody 9E10 (20) or antibody to alkaline phosphatase or to β-galactosidase was added and the samples were incubated at 4°C for 1 hr. Samples were centrifuged, washed twice with 0.5 ml of STEB, resuspended in the same buffer, and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin antibodies (for 9E10) or peroxidase-conjugated protein A (for antibody to alkaline phosphatase or β-galactosidase) at 4°C for 1 hr. Samples were centrifuged and washed four times with 0.5 ml of STEB.

Antigen–antibody complexes were then quantified by measuring OD₄₅₀ after one-step Turbo ELISA (Pierce).

RESULTS

Expression of huβ₂AR in E. coli. To express the huβ₂AR gene in E. coli, bacteria were transformed with pUC18-huβ₂AR, which contains the coding region of the huβ₂AR gene (15) fused to the IPTG-inducible lac promoter.

The huβ₂AR protein expressed in E. coli retained its characteristic ligand binding profile. A replica filter assay (12, 13) permitted direct identification of bacterial colonies expressing huβ₂AR. Bacterial colonies transformed with pUC18-huβ₂AR and adsorbed into nitrocellulose filters impregnated with IPTG bound [¹²⁵I]iodocyanopindolol (¹²⁵ICYP) (Fig. 1), whereas colonies transformed with vector plasmid alone did not bind ¹²⁵ICYP. Unlabeled agonists competed with ¹²⁵ICYP for binding; huβ₂ARs expressed in bacteria retained their pharmacological properties when the cells were transferred onto filters. A typical β₂ adrenergic order of potency was observed: isoproterenol > epinephrine > norepinephrine (12).

The huβ₂AR performs two main functions in human plasma membranes: it binds external ligands specifically and, as a result of the binding, activates a G protein. That the receptor expressed in E. coli retains its typical affinities for the specific ligands (11–13) and couples with Gsα (14) suggests that it adopts a configuration in the E. coli membrane similar to that found in mammalian plasma membranes.

Isolation of huβ₂AR–PhoA Fusions. We used a combination of in vivo and in vitro techniques to isolate gene fusions encoding proteins with various lengths of huβ₂AR from the N terminus joined to PhoA at the C terminus (Fig. 2). Since PhoA must be exported to the periplasm if it is to acquire enzymatic activity, fusion of PhoA to domains of huβ₂AR normally located on the periplasmic side of the membrane should result in a Pho⁺ phenotype, whereas fusions to cytoplasmic regions of huβ₂AR should yield a Pho⁻ phenotype (3).

We first generated huβ₂AR–PhoA fusions in vivo by transposition of TnphoA (17) into the huβ₂AR gene carried on pUC18. We examined 23 independent Pho⁺ huβ₂AR:TnphoA insertions and found 13 different fusion sites in the huβ₂AR molecule (Fig. 2). PhoA enzymatic activity was monitored with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate, which had been added to the solid medium. As expected, these Pho⁺ fusions joined the huβ₂AR and PhoA
coding sequences in the proper reading frame. The 13 fusion sites are clustered in the first three transmembrane domains and in the C terminus of the huβ2AR molecule.

To direct PhoA insertions in the central domains of the huβ2AR, we used pHPO (16) directly fused to the BAL-31-treated pUC18-huβ2AR construct. In this way we obtained 24 additional huβ2AR–PhoA fusions.

Use of PhoA Fusions to Study huβ2AR Topology. The fusions and their activities are shown on the sequence of huβ2AR presented as having the topological structure suggested by the hydropathy plot (Fig. 2). The PhoA activity of the fusion proteins was determined as described (5). PhoA acquires enzymatic activity only when it is translocated to the periplasm, where its intrachain disulfide bonds can form; high activity is observed when the phosphatase domain is located on the outside of the cell membrane, whereas fusions in which phosphatase sequences are located on the inside of the membrane yield low activity. Because the PhoA activities of different fusion proteins can vary substantially, they were normalized to the amount of hybrid protein made. PhoA activity and the rate of fusion protein synthesis were determined for the same culture of each strain. The rate of fusion-protein synthesis was determined by pulse labeling with [35S]methionine, immunoprecipitation, and SDS/PAGE followed by quantitation of the bands. The relative specific activity was calculated by dividing the measured PhoA activity by the rate of protein synthesis (21, 22).

On the basis of saturation curves of 125I CYP binding to bacterial suspensions, we calculated that the number of active receptors per cell was 20 (data not shown). A larger number was expected on the basis of the PhoA activity units of the fusions (23). However, the unfused huβ2AR and the long huβ2AR–PhoA fusions produce lower amounts of protein than do the short fusions. For example, the long fusion D, which fuses PhoA to the entire huβ2AR, has a rate of synthesis 20 times less than that of the short fusion A, whose joint with PhoA is in the first cytoplasmic loop; this could account for the low number of molecules measured in the binding assay. The number of molecules estimated from the PhoA activity of fusion D is in line with that calculated from 125I CYP binding to the unfused huβ2AR. The difference in protein levels between fusions A and D was reflected in a pulse–chase experiment, which also revealed that both short and long fusions were stable after a 10-min chase (Fig. 3). The difference in amount of hybrid proteins made with the two fusions was even more evident when steady-state levels were assayed by Western blot (Fig. 4).

Analysis of the Activities of the Fusions. The enzymatic activities of the huβ2AR–PhoA fusions from the third transmembrane domain to the end of the protein agree with the proposed topology of the receptor. Among these fusions, those in predicted extracytoplasmic regions or in transmembrane domains close to the periplasm show high PhoA activities (300–760 units), while fusions in predicted cytoplasmic regions or in transmembrane domains close to the cytoplasm have low PhoA activities (10–100 units). There is, however, a serious discrepancy between the proposed topol-

![Fig. 3. Pulse–chase experiment. Bacteria were pulsed for 30 sec with [35S]methionine and "chased" with unlabeled methionine for 0, 2.5, 5, or 10 min as indicated. Fusion A (Left) and D (Right) were immunoprecipitated by anti-PhoA antibodies.](image)

![Fig. 4. Immunoblotting experiment. Bacteria from fusions A, D, and C were broken by freeze–thaw treatment. Pellets [membrane plus cytoplasm (p)] and supernatants [periplasm (s)] were separately loaded for SDS/9% PAGE, transferred onto nitrocellulose filters, and probed with anti-PhoA antibodies.](image)

![Fig. 5. Trypsin-accessibility test of fusion A protein (see Fig. 2). Spheroplasts were treated with trypsin (200 μg/ml) for 30 min at 0°C. Pellets [membrane plus cytoplasm (p)] and supernatants [periplasm (s)] were then immunoprecipitated with anti-PhoA antibodies. A, fusion A; AP, full-length PhoA.](image)
fusion A was at least 20 times higher than that of the whole tagged huβ2AR and the tagged sandwich fusion. In control experiments with the same fusions, antibody to alkaline phosphatase gave the pattern expected from the PhoA activities (Fig. 6B), and antibody to the cytoplasmic protein β-galactosidase gave low background values, showing that the spheroplasts were intact.

**DISCUSSION**

huβ2AR is one of a family of receptors characterized by seven transmembrane helices (8). The topographical disposition of all eight of the hydrophilic sequences of huβ2AR expressed in mammalian cells was established with site-directed antibody and in situ analysis by indirect immunofluorescence (10). huβ2AR expressed in E. coli displays affinities for adrenergic ligands similar to those observed in eukaryotic cells (11–13) and is able to couple with Gα (14): it appears, therefore, that the essential requirements for the accurate folding of this human receptor exist in E. coli. The huβ2AR retains its ligand-binding properties when it is expressed in E. coli both as a fusion protein (with β-galactosidase, the outer membrane protein LamB, and with the periplasmic protein MalE) or as a protein by itself. The expression level for the above bacterial expression systems is not very high and may be due to an intrinsic capacity of the E. coli membrane for the receptor or to a difficulty in translation.

To investigate the membrane topology of huβ2AR expressed in bacteria, we used a combination of in vivo and in vitro techniques to isolate gene fusions encoding proteins with different lengths of huβ2AR at their N termini joined to C-terminal PhoA. Since PhoA must be exported to the periplasm if it is to acquire enzymatic activity, fusion of PhoA to domains of huβ2AR normally located on the periplasmic side of the membrane should result in a Pho+ phenotype, whereas fusions to cytoplasmic regions of huβ2AR should yield a Pho− phenotype (3).

The enzymatic activities of the fusions from the third transmembrane domain to the end of the protein are in good agreement with the topology of the receptor in eukaryotic cells. PhoA fusions to putative cytoplasmic domains or to transmembrane segments close to the cytoplasm have low PhoA activities, whereas fusions in external domains or with endpoints well into membrane-spanning segments yield high PhoA activities.

Levels of PhoA activities of fusions with N-terminal region and the second hydrophobic domain do not agree with the expected topology of huβ2AR. The data suggest that in the N-terminal fusion the orientation of the molecule is inverted and the N terminus is localized in the cytoplasm. As shown by the reactivity of the tag epitope in the immunoassay in spheroplasts (Fig. 6), the N terminus of the huβ2AR and the sandwich fusion is located outside the membrane, whereas for fusion A it is in the cytoplasm.

Fig. 7 shows a working model of huβ2AR–PhoA hybrid

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![Figure 6](image1.png)

**Fig. 6.** ELISA on spheroplasts. Bacteria expressing the tagged fusion A (Tag-A), the tagged huβ2AR (Tag-D), and the tagged sandwich fusion (Tag-S) were converted to spheroplasts and incubated with antibody 9E10 (anti-tag) (A) or with antibodies to PhoA (B). Binding is expressed by considering the sample with the highest value as 100% and normalizing the values to the amount of protein made.

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![Figure 7](image2.png)

**Fig. 7.** Model for the insertion of the huβ2AR–PhoA hybrid protein into the inner membrane. In the absence of huβ2AR C-terminal sequence, the hybrid protein localizes the N terminus in the cytoplasm, whereas in its presence the correct insertion of the protein is obtained.
protein into the plasma membrane of E. coli. The huβ2AR–PhoA hybrid proteins with fusion in the N-terminal regions of the huβ2AR insert in an inverted orientation, localizing the N terminus in the cytoplasm, whereas the presence of C-terminal segments, the N terminus is exposed to the periplasm. Results with fusions E and F, which appear to localize PhoA in the cytoplasm with inverted orientation with respect to the proposed structure, strengthen the model presented in Fig. 7. Moreover, the sandwich fusion, in which PhoA is inserted at the same amino acid position as in fusion A in an otherwise intact huβ2AR, restores the correct orientation of the N terminus, as shown by the low levels of PhoA activity and the reactivity of the tag epitope in the ELISA.

We suggest therefore that the N-terminal fragments of the huβ2AR do not provide a complete topological information and that the correct positioning of its N terminus in E. coli is dependent on the presence of C-terminal portions of the molecule. The sequences involved in the determination of the orientation should lie between the second hydrophobic domain and the second intracytoplasmic loop of the molecule. In huβ2AR, the second cytoplasmic domain is positively charged and probably helps define the correct assembly of the protein. The positive-inside rule of von Heijne states that hydrophobic sequences orient themselves in the membrane in such a way that the most positively charged end falls in the cytoplasm (28–34).

Studying the topology of the yeast α pheromone receptor (STE2) (35) in bacteria by means of STE2–PhoA fusions, we have demonstrated that the three positive charges in the first cytoplasmic loop play a fundamental role in orienting the protein in the bacterial membrane (R.M.L., A.C. and F.S., unpublished results).

It has been proposed that interactions between membrane-spanning segments can play a role in stabilizing the structure of multspanning membrane proteins or to drive dimersizations (36, 37). These interactions may be important for the proper insertion of those N-terminal transmembrane segments that contain hydrophilic amino acids (38). In the case of huβ2AR, we can hypothesize that interactions between the third transmembrane domain and N-terminal regions may help determine the proper insertion and orientation of the molecule in the cell membrane.

The involvement of multiple regions of a polytopic membrane protein in the determination of the overall topology (31) is in contrast to a linear insertion model where the most N-terminal transmembrane segments insert first in the cell membrane, and the rest of the chain simply follows the topological dictate of the first insertion. However, the linear insertion model could better describe assembly of membrane proteins into the endoplasmic reticulum (32) of eukaryotes.

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