Video Article

Transmembrane Domain Oligomerization Propensity determined by ToxR Assay

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Abstract

The oversimplified view of protein transmembrane domains as merely anchors in phospholipid bilayers has long since been disproven. In many cases membrane-spanning proteins have evolved highly sophisticated mechanisms of action.[1-3] One way in which membrane proteins can modulate their structures and functions is by direct and specific contact of hydrophobic helices, forming structured transmembrane oligomers.[4,5] Much recent work has focused on the distribution of amino acids preferentially found in the membrane environment in comparison to aqueous solution and the different intermolecular forces that drive protein association.[6,7] Nevertheless, studies of molecular recognition at the transmembrane domain of proteins still lags behind those of water-soluble regions. A major hurdle remains: despite the remarkable specificity and affinity that transmembrane oligomerization can achieve,[8] direct measurement of their association is challenging. Traditional methodologies applied to the study of integral membrane protein function can be hampered by the inherent insolubility of the sequences under examination. Biophysical insights gained from studying synthetic peptides representing transmembrane domains can provide useful structural insight. However, the biological relevance of the detergent micellar or liposome systems used in these studies to mimic cellular membranes is often questioned; do peptides adopt a native-like structure under these conditions and does their functional behaviour truly reflect the mode of action within a native membrane? In order to study the interactions of transmembrane sequences in natural phospholipid bilayers, the Langosch lab developed ToxR transcriptional reporter assays.[9] The transmembrane domain of interest is expressed as a chimeric protein with maltose binding protein for location to the periplasm and ToxR to provide a report of the level of oligomerization (Figure 1).

In the last decade, several other groups (e.g. Engelman, DeGrado, Shai) further optimized and applied this ToxR reporter assay.[10-13] The various ToxR assays have become a gold standard to test protein-protein interactions in cell membranes. We herein demonstrate a typical experimental operation conducted in our laboratory that primarily follows protocols developed by Langosch. This generally applicable method is useful for the analysis of transmembrane domain self-association in *E. coli*, where β-galactosidase production is used to assess the TMD oligomerization propensity. Upon TMD-induced dimerization, ToxR binds to the *ctx* promoter causing up-regulation of the *LacZ* gene for β-galactosidase. A colorimetric readout is obtained by addition of ONPG to lyzed cells. Hydrolytic cleavage of ONPG by β-galactosidase results in the production of the light absorbing species o-nitrophenolate (ONP) (Figure 2).

Video Link

The video component of this article can be found at https://www.jove.com/video/2721/

Protocol

1. Cloning Considerations

1. Commercially prepared oligonucleotides representing the TMD of interest flanked by NheI and BamHI restriction sites and 5'-phosphorylated can be ligated into pTox7 (modified in our laboratory by insertion of one base pair directly after the BamHI restriction site[14]) (Figure 3) digested sequentially with BamHI and NheI. An example oligonucleotide is shown below:
   5'ctagcTMDSEQUENCEg3'
   3' gTMDSEQUENCEcctag5'

The TMD sequence should be 12-24 residues (shorter sequences will presumably be elongated by vector encoded hydrophobic residues). In order to investigate the interface, four variants of the TMD design should be created where sequential residue insertions and concomitant residue deletions result in rotation of the TMD relative to ToxR.[15,16] Finally, the arabinose concentration should be varied between 0.001 and 0.01% (w/v) to identify the concentration where maximum differences in β-galactosidase signals between different TMD sequences are observed; testing different expression levels is recommended to identify conditions under which different affinities can be distinguished best. In addition to arabinose and antibiotics, 0.4 mM IPTG can be used to enhance differences of affinities between different TMDs. The ToxR measurement should be performed at least in quadruplicate. The whole procedure should be repeated at least three times with different plasmid transformations.

2. Growth of Bacterial Cultures

1. Gently thaw FHK12 competent cells (200 µl) on ice and transfer into a 15 ml culture tube. Add plasmid DNA (200 ng) and incubate the cells on ice for 30 min.
2. Heat-shock cells by incubation for 90 s at 42 °C, followed by incubation on ice for 2 min.
3. Add SOC media (800 µl) and incubate the samples at 37 °C with shaking (300 rpm) for one h.
4. Inoculate 5 ml LB media with chloramphenicol (30 µg/ml) and arabinose (0.0025% w/v) with 50 µl of the transformation mixture in 15 ml culture tubes in triplicate. Incubate samples at 37 °C with shaking (300 rpm) for 20 h. (Alternatively 5 µl of culture can be used to inoculate 100 µl of medium in a 96-well plate. This method is useful when dealing with large numbers of samples, although errors will be slightly higher. In order to avoid evaporation, which would lead to error, fill the outermost wells with media, but do not use them for samples. Finally, double-wrap the joint between the lid and the plate with parafilm).

3. Measurement of β-galactosidase Activity

1. Preheat the plate-reader to 28 °C.
2. Transfer the Z-buffer into a reservoir with a large pipette tip, making sure to only take up the upper (aqueous) layer. Transfer 100 µl of freshly prepared Z-buffer/chloroform to the wells of a 96-well plate. Transfer 5 µl of each culture into the wells of the plate in quadruplicate. Omit the culture from four wells which will serve as the blank.
3. Measure the OD_{595} of the plate to determine cell density.
4. Add 50 µl of Z-buffer/SDS to all wells of the plate. Shake the plate in the plate-reader for 10 min to lyze the cells. Make sure the cell suspensions are clear after lysis and repeat the shaking step if required. Incomplete lysis suggests the Z-buffer/chloroform was not freshly prepared.
5. Add 50 µl freshly prepared Z-buffer/ONPG to all wells and return the plate to the plate reader and measure OD_{405} every 30 s for 20 min.
6. Calculate β-galactosidase activity using the following equation (remembering to subtract the blank). The ratio of OD_{405}/min should be calculated using all data points in the OD_{405} range 0.0 to 1.0 using a linear model fit.

$$MillerUnits = \frac{OD_{405}/\min}{OD_{595}} x1000$$

Miller units differ sometimes when recorded on different days. Therefore, a reference construct like GpA should be measured in each test. Its values can be used for normalization of ToxR values.

4. Control for Protein Expression

1. Perform Western blotting to verify even protein expression between constructs. Combine 50 µl of the triplicate cultures and centrifuge (2000 rpm, 4 min) in a microcentrifuge. Remove the supernatant by pipetting and resuspend the residual pellet in 2 x sample loading buffer.
2. Load 7.5 µl on a standard 8% gel and carry out electrophoreses at 125 V for 1 hour 5 min. After transfer, incubate with anti-MBP HRP-conjugated antibody and visualize; the chimeric protein is observed at approximately 70 kDa with some degradation products sometimes seen around 48 kDa. Endogenous MBP is also observed at 45 kDa (see Figure 5).

5. Control for Proper Membrane Insertion

A cell line deficient in maltose binding protein is used to assess proper membrane insertion of the chimeric TMD construct. When grown on minimal media with maltose as the sole carbon source, only cells expressing a membrane-integral expression product with maltose binding protein correctly located to the periplasm are able to grow.

1. Transform PD28 cells (as described for FHK12 cells) and inoculate 2 ml of LB medium. Grow the cells at 37 °C with shaking (300 rpm) overnight.
2. Pellet the cells by centrifugation at 3500 rpm, 10 min, 4 °C and wash by resuspension in PBS (2 ml) by gentle pipetting with a large tip or gentle vortexing. Pellet the cells (as above), wash with PBS for a second time, pellet and finally resuspend in PBS (1 ml).
3. Use 25 µl of resuspended cells to inoculate 5 ml minimal media in triplicate and incubate at 37 °C with shaking (300 rpm). Take OD_{595} readings between 15-25 h, approximately every 2 hours by transferring 200 µl of each sample into a 96-well plate and reading using the plate-reader.

6. Representative Results:

An example of the use of the ToxR transcriptional reporter assay to analyze the oligomerization propensity of transmembrane domains in shown in Figure 4. Previously we have investigated the oligomerization of transmembrane domains from the multispanning membrane-integral protein latent membrane protein-1 (LMP-1) by various techniques, including ToxR.[14] Transmembrane domain five (TM5) was shown to exhibit a strong propensity to oligomerize; this is demonstrated by high Miller Units, comparable to the positive control, GpA, a well-established dimerizing sequence. A deleterious mutation in TM5, D150A, reduces the ability of the sequence to oligomerize. LMP-1 TM1 does not significantly oligomerize and exhibits a very low Miller Unit signal, just above the signal for blank, non-transformed FHK12 cells.

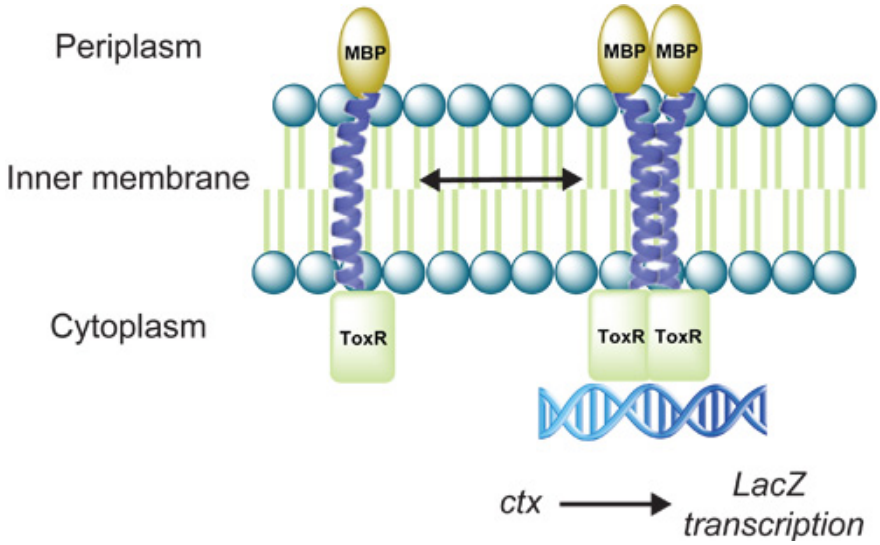

Figure 1. Cartoon depicting the ToxR reporter assay. Transmembrane domain (TMD) driven oligomerization results in dimerization of ToxR and activation of *LacZ* transcription. The gene product of *LacZ*, β-galactosidase can be quantified as a measure of the propensity of a TMD to oligomerize.

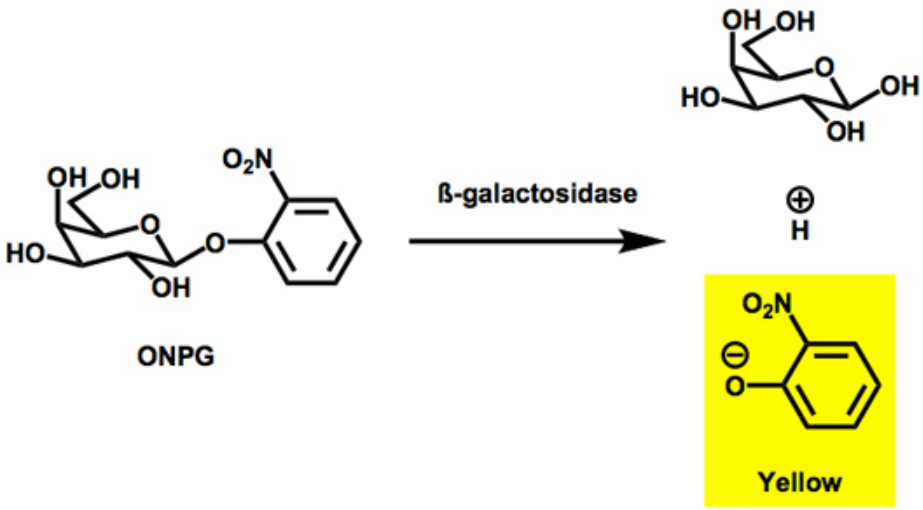

Figure 2. The hydrolytic cleavage of ONPG by β-galactosidase results in the production of the light absorbing species *o*-nitrophenolate (ONP).

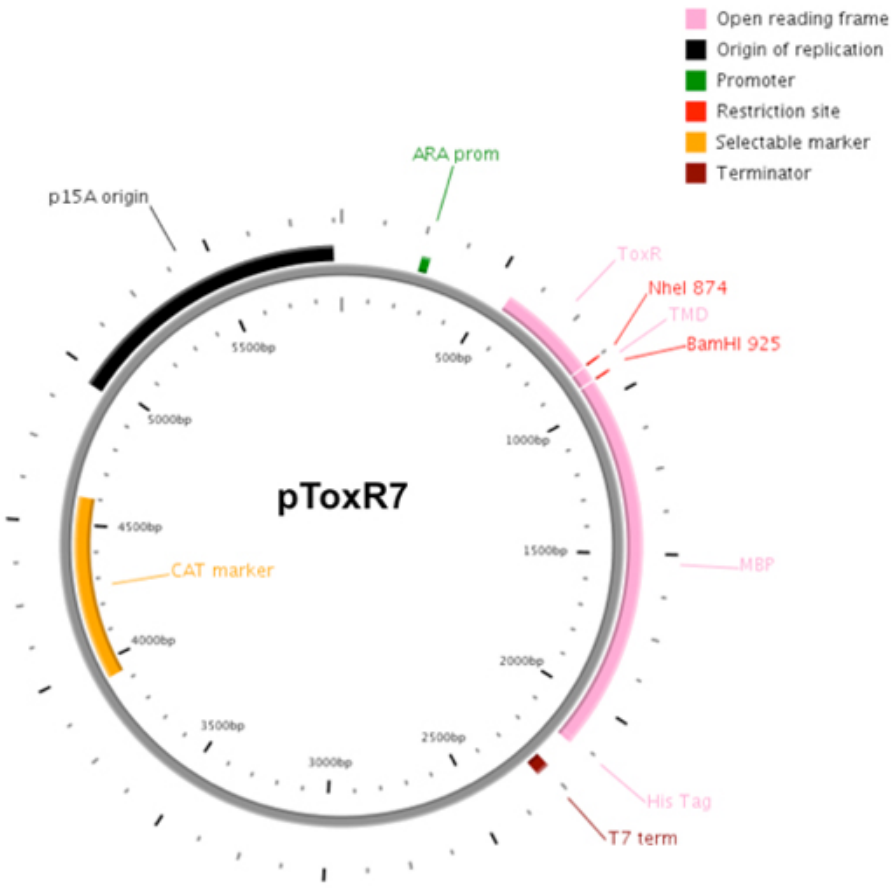

Figure 3. Plasmid map of pToxR7.

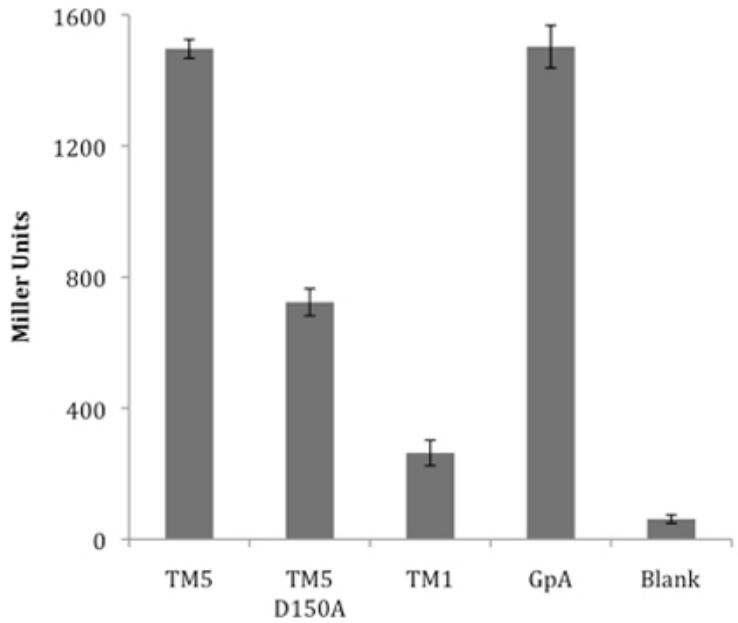

Figure 4. Representative ToxR transcriptional reporter assay analysing the oligomerization propensity of latent membrane protein-1 transmembrane domains. Transmembrane domain 5 (TM5) oligomerizes strongly, whilst transmembrane domain 1 (TM1) exhibits only a weak interaction. Mutation D150A in TM5 significantly reduces its ability to oligomerize. GpA is included as a positive control sequence for strong dimerization. Blank represents untransformed FHK12 cells.

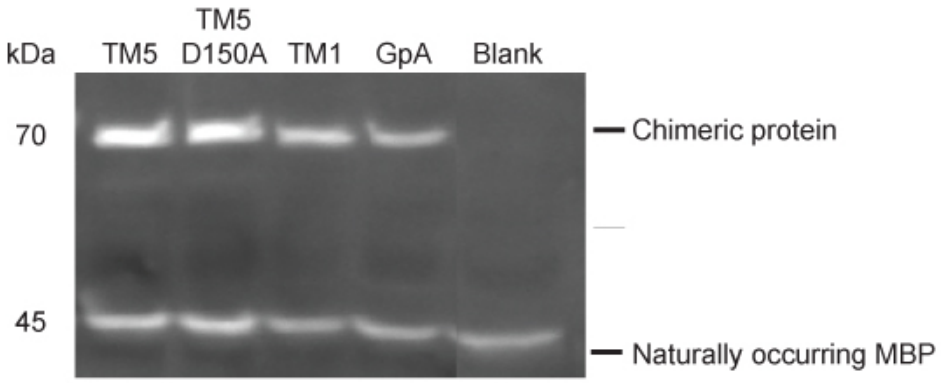

Figure 5. Western blot for protein expression.

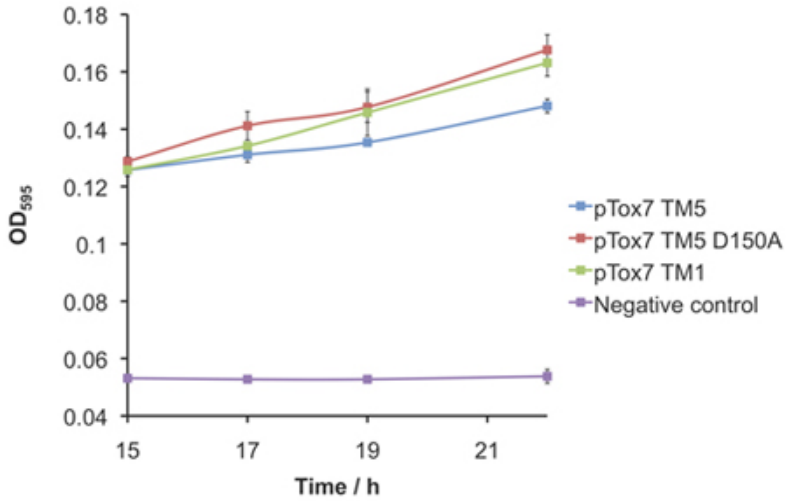

Figure 6. PD28 complementation assay to control for correct membrane insertion to the periplasm. Negative control represents a construct deficient in maltose binding protein.

Discussion

The ToxR transcriptional reporter assay is a facile way to identify transmembrane sequences with the potential to oligomerize. Since the interactions are occurring within the bacterial inner membrane, this assay circumvents the issues associated with the validity of studying systems in membrane-mimetic environments. Given that cloning of multiple TMDs into a single plasmid can readily be done in parallel and the entire assay can be carried out in 96-well plate format, this assay can be used for high throughput analysis of large numbers of protein sequences.[17] Once an interaction has been detected, the key functional residues can be interrogated by mutational analysis, allowing mapping of the structural features involved. In many cases, crystallographic analysis of transmembrane proteins is problematic, requiring alternative tools such as the ToxR assay to establish the molecular basis of function.

Disclosures

No conflicts of interest declared.

Acknowledgements

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