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Multi-Tox: Application of the ToxR-transcriptional reporter assay to the study of multi-pass protein transmembrane domain oligomerization

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ABSTRACT

ToxR-based transcriptional reporter assays allow the strength of transmembrane helix interactions in biological membranes to be measured. Previously, these assays have only been used to study single-pass transmembrane systems. To facilitate investigation of polytopic transmembrane domain (TMD) oligomerization, we applied the ToxR methodology to the study of multi-pass TMD oligomerization to give 'Multi-Tox'. Association propensities of the viral oncoprotein, latent membrane protein-1 (LMP-1), and the *E. coli* membrane-integral diacylglycerol kinase (DAGK) were studied by Multi-Tox, highlighting residues of particular mechanistic importance. Both homo- and hetero-oligomerizations were studied.

1. Introduction

Despite the ever-increasing interest in membrane protein structures and functions, interactions mediated by transmembrane domains (TMDs) remain poorly understood. Homo- and hetero-oligomerizations via TMDs play key roles in the function of many integral membrane proteins of therapeutic interest, such as G protein-coupled receptors (GPCRs) [1] and transporters [2]. The groups of Langosch and Engelman developed the ToxR/ToxCAT assays which enable measurement of the strength of transmembrane helix interactions in the natural phospholipid environment of the *E. coli* inner membrane [3,4]. TMD-induced oligomerization of a chimeric protein comprised of maltose binding protein (MBP) for localization to the periplasm fused to the TMD of interest and cytosolic ToxR, results in proximity-induced dimerization of ToxR. Dimeric ToxR binds to the *ctx* promoter and downstream signaling can be measured by a variety of methods (Fig. 1a). More recently, the teams led by Engelman and Dixon demonstrated that signaling via ToxR can be driven by not only simple dimerization but also higher order association (e.g. trimerization) [5,6] of the TMD. In the past decade, further work by the groups of Engelman and Langosch as well as others (such as Shai, Dixon, Flemming, MacKenzie, Schneider, and DeGrado) have significantly improved the ToxR family of assays that have become a crucial tool in the elucidation of function of a wide range of integral membrane proteins [7–11]. However, previously only single-pass transmembrane systems have been investigated using ToxR/ToxCAT assays. Since 53% of human membrane proteins are predicted to have more than one transmembrane helix [12], an assay designed to study multi-pass TMD interactions will provide valuable functional information on proteins that are inherently difficult to study by conventional techniques. To facilitate investigation of membrane protein–protein interactions, we applied the ToxR transcriptional reporter assay to the study of polytopic TMD oligomerization in biological membranes and named this extension of the methodology 'Multi-Tox' (Fig. 1b).

2. Materials and methods

2.1. Plasmids

pTox7 [13] (gifted from D. Langosch, Technische Universität München, Germany) was modified by insertion mutagenesis to add one base (t) directly following the BamHI restriction site. pTox7 TM5 and pTox7 TM1 were created by ligating double stranded synthetic oligonucleotides into the NheI/BamHI sites of modified pTox7. Double stranded oligonucleotides encoding TM5 and TM1 were obtained from Integrated DNA Technologies (IA, USA). pTox7 TM5 D150A was created using standard site directed mutagenesis with a commercially available Stratagene Quikchange II kit (Agilent, CA, USA). pTox7 TMD456 was prepared by ligating two sequential double stranded synthetic oligonucleotides (Integrated DNA Technologies, IA, USA) into the NheI/BamHI sites of modified pTox7 in one pot. TMD inserts for TMD123, TMD234, TMD345 and DAGK were prepared commercially by Genewiz (NJ, USA), flanked by

Abbreviations: DAGK, diacylglycerol kinase; DN, dominant-negative; EBV, Epstein-Barr Virus; GpA, Glycophorin A; GPCR, G protein-coupled receptors; LMP-1, latent membrane protein-1; MBP, maltose binding protein; ONPG, *ortho*-nitrophenyl β-galactoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMD, transmembrane domain

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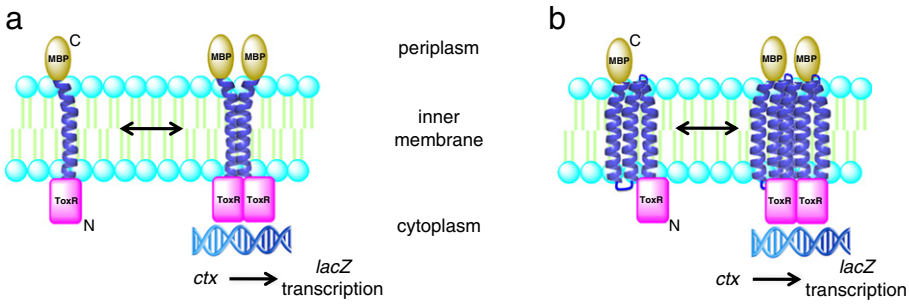

Fig. 1. Cartoon representations of (a) ToxR and (b) Multi-Tox assays. Maltose-binding protein (MBP) locates to the periplasm, forcing a parallel orientation of TMDs in the membrane. TMD-driven oligomerization results in dimerization of ToxR and activation of signaling by the *ctx* promoter.

NheI and BamHI restrictrion sites, in pUC57. After restriction digest from pUC57, exerts were ligated into modified pTox7. All transmembrane sequences were codon-optimized for *E. coli*. TMD mutants were created using standard site directed mutagenesis with a commercially available Stratagene Quikchange II kit (Agilent, CA, USA).

2.2. ToxR assay

ToxR plasmids (200 ng) were transformed into 200 µl FHK12 competent cells (kindly provided by D. Langosch, Technische Universität München, Germany) with heat shock at 42 °C for 90 s and incubation on ice for 2 min, followed by addition of 800 µl SOC medium and incubation with shaking at 37 °C for 1 h. An aliquot of the transformation mixture (50 µl) was used to inoculate 5 ml LB broth in the presence of arabinose (0.0025%) and chloramphenicol (30 µg/ml) in triplicate. Cultures were incubated with shaking at 37 °C for 20 h and β-galactosidase activity was measured using a Beckman Coulter DTX 880 plate reader (Beckman Coulter, CA, USA) as follows: 5 µl of each culture was transferred in quadruplicate to the wells of a Costar 3596 polystyrene 96-well plate (Corning, NY, USA) containing 100 µl Z buffer/chloroform (1% β-mercaptoethanol, 10% chloroform, 89% A buffer: 1 M sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$ pH 7.0). Cell densities were recorded by measuring OD_{595}. Cells were lysed by addition of 50 µl Z sodium dodecyl sulfate (SDS) in Z buffer (1.6% w/v) and shaking at 28 °C for 10 min. To the resultant mixture, 50 µl *ortho*-nitrophenyl β-galactoside (ONPG) in Z buffer (0.4% w/v) was added and β-galactosidase activity was measured by monitoring the reaction at 405 nm for a period of 20 min at intervals of 30 s at 28 °C. Miller Units were calculated using the following equation:

$$\textit{Miller Units} = \frac{OD_{405}\,/\,\min}{OD_{600}} \times 1000$$

2.3. DN-ToxR assay

Both pTox6 [14] and plasmid control [13] were kindly provided by D. Langosch, Technische Universität München, Germany and pTox6 was modified by insertion of a single base (t) after the BamHI restriction site using standard Quikchange mutagenesis protocols. The pTox6 variant containing LMP-1 TMD456 was prepared as described for pTox7 above. DN-assays were carried out in an analogous manner to ToxR assays except double volumes of FHK12 competent cells and SOC were used for dual transformations and selection was achieved by addition of chloramphenicol (30 µg/ml) and kanamycin (33 µg/ml).

2.4. Control experiments

Western blotting was performed with antiserum recognizing the maltose binding protein moiety of the constructs (New England Biolabs, MA, USA). The chemiluminescence output was captured using either X-ray film or a CCD camera imaging device and the 60–70 kDa region of the blot is shown. The color of images captured using X-ray film was inverted. Controls for membrane integration were performed by transforming PD28 cells with the indicated plasmids. Cells were grown in minimal media with 0.4% maltose as the sole carbon source. Cell density was monitored by OD_{595} and corresponds to the efficiency of membrane integration. PD28 cells were kindly provided by D. Langosch, Technische Universität München, Germany.

3. Results and discussion

3.1. Multi-Tox analysis of membrane-integral diacylglycerol kinase oligomerization

In order to study multi-pass TMD oligomerization, chimeric proteins comprised of maltose binding protein (MBP) for location to the periplasm, the polytopic TMD of interest and the DNA-binding domain of ToxR for signaling were expressed in the *E. coli* indicator strain, FHK12. TMD-driven oligomerization facilitates dimerization of ToxR, leading to transcriptional activation of the *ctx* promoter and expression of β-galactosidase. The level of β-galactosidase correlates to the oligomerization propensity of the TMD (Fig. 1b).

A well-studied triple-pass protein, membrane-integral diacylglycerol kinase (DAGK) from *E. coli*, was chosen as a model system to validate the Multi-Tox assay. Previous biochemical and structural analyses have assessed various DAGK TMD residues for their ability to disrupt oligomerization [15]. A vector for expression of a chimeric protein containing the entire DAGK TMD (MBP–DAGK TMD–ToxR) was constructed and standard site-directed mutagenesis was performed to introduce six individual point mutations in the transmembrane region (Fig. 2c). Expression of the chimeric proteins in *E. coli* and quantification of β-galactosidase levels was performed. Multi-Tox analysis of oligomerization of the entire DAGK TMD (three-pass) agreed with earlier findings reported by Sanders and co-workers [15]; the A100C mutation has been identified to destabilize trimerization and this was indeed observed in our assay, whilst mutations reported to have modest or little effect on trimerization (E69C and N72C) showed no significant reduction of oligomerization (Fig. 2a). Interestingly, K94C, a mutation for which the effect on trimerization was previously described as 'unclear,' did affect oligomerization propensity in our assay, suggesting potential oligomerization/structure stabilization roles played by this lysine residue at the membrane/aqueous interface. A double mutant of K94C and A100C showed a small additive effect on the association of the DAGK TMD, reconfirming the previously identified importance of A100 in the third transmembrane helix and highlighting a role for neighboring K94. To further understand the effects of simultaneously mutating residues on different DAGK TM helices, a triple mutant (E69C, K94C, A100C) was constructed. The E69C mutation exerted only a small negative change in oligomerization propensity, both as a single mutant or as part of the triple mutant. These results agree well with the previously reported X-ray crystal structure of DAGK [15] where the E69C