BLaTM 2.0, a Genetic Tool Revealing Preferred Antiparallel Interaction of Transmembrane Helix 4 of the Dual-Topology Protein EmrE

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Abstract

Parallel and antiparallel transmembrane helix–helix interactions support the folding and non-covalent assembly of many integral membrane proteins. While several genetic tools are currently in use to study parallel transmembrane helix–helix interactions, antiparallel associations have been difficult to determine. Here, we present a novel genetic approach, termed BLaTM 2.0, which can be used in combination with the recently presented BLaTM 1.2 to compare the efficiency of antiparallel and parallel transmembrane domain (TMD) interactions in a natural membrane. In a practical application of the BLaTM system, we find that the antiparallel interaction of TMD4, the known dimerization domain of the dual-topology small multidrug transporter EmrE, is sequence-specific and much stronger than the parallel one. This suggests that TMD4 has evolved to favor the formation of dual-topology EmrE dimers over single-topology dimers.

Introduction

Folding and oligomerization of integral membrane proteins are frequently supported by sequence-specific interactions of their helical transmembrane domains (TMDs) [1–4]. In the case of single-span membrane protein dimers or multimers, TMDs usually interact in a parallel homotypic or heterotypic fashion and thus share their respective N_{in} (Type 2) or N_{out} (Type 1) transmembrane topologies. In contrast to that, the up-and-down topology of multispan proteins mandates that neighboring TMDs within a subunit pack to each other via antiparallel heterotypic interaction, while multimerization can be mediated by parallel or antiparallel TMD association.

Several bacterial hybrid systems have been developed for the investigation of parallel TMD interactions within the inner *Escherichia coli* membrane. These genetic tools convert the efficiency of interaction into graded levels of reporter gene expression. They include the ToxR transcription activator system [5], which was developed into a number of variants including TOXCAT [6], dsTβL [7], TOXGREEN [8], Multi-Tox [9], and dominant-negative versions [10,11]. DN-AraTM is another dominant-negative assay based on AraC [12]. In the GALLEX assay, TMD–TMD association dimerizes the LexA transcription factor, thus downregulating reporter gene expression [13]. In the BACTH system, interaction drives the reconstitution of cAMP-producing adenylate cyclase, supporting reporter gene expression [14–16]. The most recent addition to this tool kit is BLaTM, where the complementation of periplasmically located β-lactamase fragments, which are genetically fused to interacting TMDs, confers ampicillin resistance to expressing cells [17].

While the original ToxR-based methods can only report homotypic TMD associations, the dominant-negative ToxR variants and DN-AraTM, BACTH, GALLEX, and BLaTM can also measure heterotypic interactions. However, all these assays exclusively address parallel TMD interactions. To our knowledge, no genetic tool has been reported yet that enables studying antiparallel TMD interactions.

The small multidrug resistance protein of *E. coli* (EmrE) represents a rare case where homodimerization of a multispan protein rests on an antiparallel TMD interaction, since both subunits assume opposite

transmembrane topologies [18,19]. After much controversy [20], the dual topology of EmrE was proven by electron microscopy [21] connected to molecular modeling [22], biochemical studies [23], X-ray crystallography [24], solution NMR spectroscopy, fluorescence resonance spectroscopy [25], and solid-state NMR [26]. During EmrE biogenesis, roughly equal fractions of subunits are membrane-inserted with N_{in} or N_{out} topology, respectively, and the dual topology arises when subunits with different topologies dimerize [27]. The structural studies revealed that EmrE assembly results from the antiparallel homodimerization of TMD4, while TMDs 1–3 bind and translocate the substrate [21–26]. To the best of our knowledge, a crucial, yet open question in the field is whether the affinity of the antiparallel TMD4 interaction exceeds that of its parallel interaction. Answering this question would reveal if TMD4 has specifically evolved to support the formation of the dual-topology dimer.

Here, we assessed parallel and antiparallel EmrE TMD4 associations using an expanded BLaTM system. Our results show that the antiparallel interaction is strongly favored over the parallel one and depends on residues located in the TMD4–TMD4 interface of the EmrE dimer structure.

Results

Design of the BLaTM system

In BLaTM, an N-terminal fragment of β-lactamase (N-BLa) functionally complements the C-terminal fragment (C-BLa) in the *E. coli* periplasm, if both fragments are fused to TMDs interacting within the inner membrane (Fig. 1). Since β-lactamase breaks down ampicillin, the affinity of the TMD interaction is reflected by the ampicillin resistance of expressing cells. This resistance is quantified as the dose of ampicillin concentration permitting the survival of 50% of the cells (LD_{50}). In the previously described BLaTM versions 1.1 and 1.2, a pair of expression plasmids encodes hybrid proteins that consist of either N-BLa or C-BLa, the TMD of interest and superfolder green fluorescent protein (sfGFP), under the control of the pBAD promoter. An N-terminal cleavable signal peptide (which differs between versions 1.1 and 1.2) ensures the N_{out} topology of both hybrid proteins, such that only parallel TMD interactions can result in β-lactamase reconstitution (Fig. 1 and Fig. S1) [17].

In order to investigate antiparallel TMD interactions, we engineered a novel version of the BLaTM system, termed BLaTM 2.0. In this system, the N_{out} N-BLa hybrid protein described above is coexpressed with a C-BLa construct where the TMD is inserted between the C terminus of a cytoplasmic ToxR domain and the N terminus of the C-BLa moiety (Fig. 1b and Fig. S1). An N-terminal ToxR domain ensures the N_{in} topology of a hybrid protein [5,6]. Consequently, membrane-integrated N-BLa and C-BLa hybrid proteins of BLaTM 2.0 assume antiparallel TMD topologies.

Parallel EmrE TMD4 interactions are of low affinity

We initially investigated the parallel EmrE TMD4 interaction using BLaTM 1.2. The efficiency of β-lactamase reconstitution does not only depend on the affinity between the fused TMDs but also on the orientation of the interacting faces of the TMD helices relative to the BLa and sfGFP domains [17]. Therefore, different frames of TMD4 within the hybrid proteins (TMD4_0 to TMD4_3 in Fig. 2a were tested. Assuming the α-helicity of a TMD, stepwise insertion of up to three additional residues at its N terminus concurrent with the stepwise deletion of three residues at its C terminus rotates the potential TMD–TMD interfaces by up to $3 \times 100°$, that is, almost a full helix turn, relative to the BLa and sfGFP domains. It has been shown previously that the stability of the EmrE dimer is diminished by the mutations G90C, I94C, G98C, I101C, and N102C within TMD4 [28]. Likewise, the TMD4 mutations G90V, L91A, L93A, I94A, G97V, and V98A abolish the dimerization of the homologous small multidrug transporter from *Halobacterium salinarum* (Hsmr) [29]. G90, L93, G97, and V98 are conserved between EmrE and Hsmr [29], and Fig. 2b depicts the location of these residues within the EmrE structure [24]. The residues (except G90 of subunit A and V98 of subunit B) are located at the EmrE TMD4 helix–helix interface.

The LD_{50} values characterizing the parallel interactions of the different EmrE TMD4 frames vary only by a small degree; they are much below the LD_{50} conferred by the well-established high-affinity glycophorin A (GpA) TMD and close to its low-affinity G83I mutant [6] (Fig. 2c, upper panel). Thus, the parallel interaction of the EmrE TMD4 is of very low affinity. Furthermore, we assessed the impact of the G90, G97, L93, and V98 on parallel self-association of TMD4_3, the construct with the highest LD_{50} value. Introducing the double mutations, G90V/G97V or L93A/V98A has only little impact on the LD_{50} value of the parallel interaction. This outcome was expected, since the value is close to the background signal.

The antiparallel TMD4 interaction is highly efficient and sequence-specific

Next, we investigated the antiparallel TMD4 interaction using BLaTM 2.0. Coexpressing both hybrid proteins harboring TMD4 confers LD_{50} values that strongly depend on its frame. Specifically, the LD_{50} of TMD4_3 exceeds those of the other orientations ~3-fold (Fig. 2d, upper panel). The