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A modified, dual reporter TOXCAT system for monitoring homodimerization of transmembrane segments of proteins

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Abstract

The TOXCAT assay system developed by Russ and Engelman [TOXCAT: a measure of transmembrane helix association in a biological membrane, Proc. Natl. Acad. Sci. USA 96 (1999) 863–868] provides an in vivo means of selecting for and evaluating the strength of interaction between identical transmembrane α-helices. In the course of utilizing TOXCAT to study the architecture of a sodium channel $hNa_V1.5$, an apparently strong dimerization of two of its putative transmembrane segments was revealed. Following random mutagenesis of these regions, several amino acids critical for the observed dimerizations were identified. In order to develop a more efficient means of isolating mutations which specifically disrupt dimerization of these transmembrane segments without affecting their membrane-targeting properties, we developed a modification to the original TOXCAT design in which the C-terminal maltose binding protein moiety is replaced by the β-lactamase. We show that this assay system is capable of simultaneously monitoring the integrity of the chimeric protein, its membrane insertion activity, and the ability of the transmembrane segment under study to dimerize.
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The TOXCAT assay system has been designed to measure homooligomerization of transmembrane segments of proteins within the context of the cytoplasmic membrane of living cells. In essence, the system relies on creation and bacterial expression of a chimeric protein in which the putative transmembrane sequence of interest is inserted between an N-terminal cytoplasmic transcriptional activation domain and a C-terminal periplasmic anchor. In its original form, such a protein is expressed in *Escherichia coli* cells from the plasmid pccKAN [1], with the N-terminal cytoplasmic domain supplied by the ToxR protein and the C-terminal periplasmic moiety being contributed by maltose binding protein (MBP). Natural ToxR is a membrane-embedded transcription factor originating from the bacterium *Vibrio cholerae* whose dimerization, driven by its periplasmic domain upon external stiumuli, leads to activation of virulence-associated genes [2].

In TOXCAT constructs, the transmembrane and periplasmic domains of ToxR are replaced by test transmembrane sequences and MBP, respectively [3,1]. As a result, dimerization must be driven solely by interactions between the tm segments. Such dimerization allows the cytoplasmic transcriptional activation domains (toxR′) to interact with the ctx promoter, thereby initiating transcription of a reporter gene present in pccKAN encoding chloramphenicol acetyltransferase (CAT). The resulting level of CAT activity, reflecting the strength of interaction, can be measured directly by any of several available biochemical assays or by the resistance of bacterial cells harboring the plasmid to graded concentrations of chloramphenicol. Thus, this system constitutes a very convenient and precise tool for measuring the strength of interactions between identical tm helices. Since its inception, TOXCAT has been employed to study the contribution of specific sequence

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motifs of transmembrane proteins such as glycophorin A [1,4] and integrin aIIb [5] to their oligomerization, as well as in selection of novel motifs important for dimerization from random sequence libraries [6]. It has provided an ultimate in vivo test showing the lack of a significant contribution by the tm domain of synaptobrevin to its dimerization, which had been postulated previously based on conflicting results from in vitro experiments [7].

Expression of the *cat* reporter gene in most cases directly reflects the extent of dimerization of the tm segments under study. However, lower CAT activities upon introduction of mutations intended to disrupt such interactions could arise not only from attenuation of dimerization but also from decreased expression of the hybrid protein or its less efficient targeting into the membrane. Although the activity of MBP provides a useful control for chimera targeting, measurement of this activity is not precise and requires a specific strain and defined synthetic medium. As a result, demonstrating that a mutated tm segment efficiently inserts into the membrane often requires using other, time-consuming techniques, such as Western blotting.

Here we present a modified version of the TOXCAT system consisting of two reporter activities capable of simultaneously monitoring both expression of the chimeric protein and its insertion into the membrane in addition to measuring the extent of dimerization of the tm segment. It is especially well suited for quick identification of random mutations specifically affecting oligomerization of tm helices of proteins.

Methods

Construction of plasmids. Plasmid pVM4 was constructed by PCR amplification, using *Pfu* polymerase, of most of the starting vector, pccKAN, except for ~1kB constituting one of the repeated regions, using the primers KB101 (TGGCGGCCGCTCTCATCCGCCAAAACAGCCG) and KB102 (AAGCGGCCGCGGGCAGCGTTGGGTCCTGGCC). These primers contained added *Not*I restriction sites, which were subsequently used to circularize the amplified DNA fragment. A DNA fragment encoding the $hNa_V1.5$ IS1 segment was inserted into plasmid pVM4, resulting in plasmid pDM805. Due to the apparent dimerization of the IS1 segment, this plasmid conferred resistance to high concentrations of chloramphenicol, which could be used for selection in the next step. The *bla* marker of pDM805 was deleted by amplification of the remaining portion of its DNA using primers ML47 (AATCTAGACCCCGGCCGGGTAC CGAGCTCG) containing a *Xba*I site, and ML64 (TTTCTAGATA AAGATCTACACGACGGGGAGTCAGGC) containing *Xba*I and *Bgl*II restriction sites. The amplified DNA fragment was digested with *Xba*I and self-ligated to obtain plasmid pD641, which was subsequently digested with *Kpn*I and *Bam*HI and ligated to a DNA fragment containing the portion of the *bla* gene encoding mature β-lactamase. The latter fragment was obtained by PCR using plasmid pccKAN as a template and the pair of primers ML41 (AAGGATCCAGTTAACCGGTGGTCACCCAGAA ACGCTGGTGAAAGTA) and 42 (TTGGTACCTACCAATGCTTAA TCAGTGAGGCACC), containing added restriction sites for *Bam*HI and *Kpn*I, respectively. Subsequently, a gene conferring resistance to spectinomycin [8] was inserted between the *Xba*I and *Bgl*II restriction sites. Finally, the sequence encoding the IS1 tm segment flanked by *Nhe*I and *Bam*HI sites was replaced by a DNA fragment containing the kanamycin resistance marker from plasmid pccKAN, yielding the final construct pML27.

Random mutagenesis. was performed by error-prone PCR using *Taq* polymerase in the presence of Mn^{2+} ions and varying concentrations of Mg^{2+} [9]. In initial experiments, we used pairs of primers specific to and complementary to the ends of sequences encoding the mutagenized tm segment IVS1: ML11 (TTGCTAGCATATTCGACATTGTGACC AAGC) and ML10 (CCGGATCCTCTCCACCATCATGGTCACCAT). With this primer set, mutations could be generated only in the central portions of the tm sequence, encompassing approximately one-third of their lengths. Subsequently, a second pair of primers, ML84 (GCTCCA AACTTGGGGAATCGAGCTAGC) and ML85 (TGGGTGACCAC CGGTTAACTGGATCC), complementary to sequences immediately adjacent to DNA coding for tm segments, was used to enable all of the tm sequence to be analyzed.

Screening methods. Following transformation *E. coli* cells were plated on LB agar supplemented with spectinomycin alone or with both spectinomycin and ampicillin at concentrations of 50 and 25 μg/mL, respectively. Subsequently, the clones obtained were grown in 100 μL liquid culture in a microtiter plate. Three to four microliters of this culture was spotted on agar supplemented with ampicillin at a concentration of 100 μg/mL and on plates containing varied concentrations of chloramphenicol (e.g., 50, 100, and 150 μg/mL). Clones growing on ampicillin were diluted so as to obtain single colonies and plated on agar containing 25 μg/mL of this antibiotic.

DNA sequencing. was performed by the Biopolymer Facility at Roswell Park Cancer Institute, Buffalo, NY.

Results and discussion

The initial goal of the work presented here was to assess the feasibility of using the TOXCAT principle to probe for interactions between tm segments of more complex integral membrane proteins as a means of gaining better insight into their overall architecture. We chose the voltage-gated sodium channel $Na_V1.5$, whose ion conducting α-subunit consists of four homologous domains numbered I–IV, each containing six putative transmembrane segments S1–S6 in addition to a re-entrant loop between S5 and S6 in each domain. All four pairs of S5–S6 regions are thought to cluster in the center of the molecule and to constitute the conductance pore through which Na^+ ions pass, whereas the S1–S4 segments provide the voltage sensor which regulates the gating mechanism [10].

In our initial experiments, we probed three putative tm segments of channel domain IV: S1, S3, and S5, all of which are oriented with their N-termini facing the cytoplasm. Following insertion of their coding sequences into TOXCAT chimeras, we found that the IVS3 and IVS5 segments conferred resistance of host *E. coli* to only rather low concentrations of chloramphenicol, suggesting that their ability to dimerize is limited at best, and most likely lacks any biological significance. In contrast, clones containing the IVS1 tm segment were consistently able to grow in liquid medium containing the antibiotic at concentrations of 60 μg/mL. Subsequent analyses of S1 tm segments from channel domains I, II, and III revealed that IS1 likewise conferred a very high level of chloramphenicol resistance, whereas IIS1 and IIIS1 did not. That S1 segments from different domains differ in their ability to dimerize is perhaps not surprising, since sequence conservation between domains is quite low. In comparison, S1 segments from different species and different subtypes of sodium channels are

highly conserved *within* a given domain. In contrast to IS1 and IVS1, none of the S3 or S5 segments we analyzed gave rise to chloramphenicol-resistant clones.

Despite the apparent dimerization detected in the TOXCAT assay, the amino acid sequences of IS1 and IVS1 do not seem to possess any readily identifiable motifs characteristic of dimerizing tm segments, such as the GXXXG motif originally selected from a randomized library by Russ and Engelman [6]. Therefore, in order to better define the amino acids which might play a role in dimerization of these S1 segments, we chose to randomly mutagenize both segments and screen for clones displaying decreased chloramphenicol resistance. Random mutagenesis of tm segments resulting in disruption of homodimerization, manifested by a decreased resistance to chloramphenicol, required an efficient and rapid selection method to discriminate against mutations decreasing the ability of these segments to insert into the membrane. Moreover, as we experienced during our initial attempts, the process of mutagenesis and subsequent insertion of DNA fragments into the pccKAN vector often resulted in a relatively high proportion of chloramphenicol sensitive clones due simply to deletions, frameshift mutations, and plasmid rearrangements. Some of these undesirable outcomes are likely ascribable to sequence repeats present in pccKAN. Because the C-terminal maltose binding protein moiety present in the chimera cannot be easily used for direct selection, we sought an alternative means of selecting for intact and membrane-targeted chimeras while screening for mutations which specifically disrupted dimerization.

β-Lactamase appeared to offer an ideal reporter activity for this purpose. Because it acts on its substrate when present in the periplasmic space of the host, this enzyme has been successfully employed as a marker in determining the topology of a number of polytopic membrane proteins (e.g., see [11,12]). Furthermore, β-lactamase allows for facile discrimination between lack of expression of the intact hybrid protein and its inability to insert into the membrane. A chimera unable to cross the membrane cannot protect single cells from ampicillin, but in a dense culture placed on antibiotic-containing agar the enzyme is released from the cytoplasm of lysing cells, allowing some bacteria to survive [13,14].

We therefore replaced the sequence encoding MBP with that of mature β-lactamase in the vector pML27, derived from pccKAN. Since β-lactamase activity serves as a selective marker in pccKAN, it was necessary to simultaneously replace the *bla* cassette with an alternative resistance marker. For this purpose, we chose the *aad9* gene [8] which confers resistance to spectinomycin. In addition, we deleted a ~1.0 kbp direct repeat present between positions 6686 and 7687 of pccKAN, as described under Methods.

Upon cloning of tm segments of the sodium channel $Na_V1.5$ into pML27, we first tested new chimeric proteins containing the β-lactamase moiety at their C-termini for their ability to confer ampicillin resistance. When spotted in the form of a dense liquid culture, bacteria could grow on agar containing as much as 250 μg/mL of this antibiotic. However, diluted suspensions of cells leading to formation of single colonies were able to survive concentrations of ampicillin no higher than 25 μg/mL. In contrast to this large difference in ampicillin sensitivity, resistance to chloramphenicol did not differ from that obtained when the same tm segments were encoded in the pccKAN context. Differential ampicillin resistance can likely be explained by the possibility that β-lactamase being a part of larger hybrid protein, tethered closely to the cytoplasmic membrane, may have significantly lower activity compared to the natural enzyme, which is able to freely diffuse within the periplasmic space, effectively decreasing the level of protection a single cell can afford against this antibiotic. In contrast, in a dense population of bacteria ampicillin can be inactivated more efficiently, especially given the likelihood of enzyme release from cells which are lysed at high concentration of this antibiotic.

Randomly mutagenized tm segments S1 of domains I and IV of human $Na_V1.5$ were inserted into pML27, and following transformation bacteria were directly selected on agar containing 25 μg/mL ampicillin in order to restrict analysis to clones producing intact and functional membrane-targeting chimeras. Alternatively, if the effect of mutations on membrane insertion needed to be evaluated, transformants were initially selected on spectinomycin. These were subsequently probed for resistance to chloramphenicol as well as for resistance to 25 μg/mL ampicillin for single colonies and to 200 μg/mL for a dense suspension. The latter test was performed to discriminate between intact chimeras unable to insert into the membrane and the clones bearing frameshift mutations or major rearrangements of the plasmid. In the course of mutagenesis, we obtained 23 clones exhibiting decreased resistance phenotypes, 10 in IS1 and 13 in IVS1. As in most experiments transformants were initially selected on ampicillin, the majority of mutants retained their ability to insert into

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A A V K I L V H S L F N M L I M C T I L T N C V F M A
          K H   K P

I F D I V T K Q A F D V T I M F L I C L N M V T M M V E
              V G P T   S                   K
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Fig. 1. Amino acid sequences of the putative tm segments IS1 and IVS1of the sodium channel $hNa_V1.5$. Underneath are shown single replacements of amino acids generated by random mutagenesis and resulting in increased chloramphenicol sensitivity while retaining the wild type resistance of single colonies to ampicillin.

the membrane while showing loss of chloramphenicol resistance to varying degrees. Among these, 10 clones had single amino acid replacements within putative tm domains (see Fig. 1), while eight had 2–3 substitutions within these same sequences.

Among the clones originally selected on spectinomycin, one contained a large, in-frame deletion of 18 (out of 28) amino acids within the tm segment. This clone was resistant to high concentrations of ampicillin when a dense culture was spotted on agar, indicating the presence of a chimeric protein having a functional β-lactamase domain at its C-terminus. As expected, this clone was unable to grow at all concentrations of chloramphenicol tested and was also sensitive to as little as 25 μg/mL ampicillin when plated as single colonies. Presumably, this is due to the absence of a sufficiently long stretch of hydrophobic amino acids to form a membrane-embedded helix. Importantly, this result validates the dual function of the *bla* reporter in discriminating between expression of full-length protein and insertion of the chimera into the membrane.

In terms of efficiency, these results are in stark contrast to earlier experiments performed in our laboratory, where dimerization of transmembrane segments of human epithelial sodium channel subunits was investigated utilizing the original TOXCAT system (Blumenthal and Combs, unpublished). In that study the amino-terminal transmembrane region of the α-subunit was found to support dimerization, whereas the corresponding regions from the β- and γ-subunits did not. This observation is consistent with models of this protein which feature subunit stoichiometries of 2:1:1 or 3:1:1 [15]. The homo-oligomerizing amino-terminal tm segment of the channel α-subunit was then mutagenized and screened for sensitivity to chloramphenicol. Among the 68 clones sensitive to this antibiotic we obtained, 63% and 20%, respectively, exhibited major plasmid rearrangements and deletions causing frame-shifts. Only 8% of clones had point mutations, and even among these, an unknown number might have resulted in inhibition of membrane insertion. These data highlight the inherent difficulty of using TOXCAT coupled to random mutagenesis to identify residues essential to dimerization in a high-throughput fashion.

The utility of the TOXCAT system as a probe for homodimerization of transmembrane regions has been demonstrated in a number of systems [1,4–6]. However, the fact that TOXCAT cannot easily discriminate between the failure of a test sequence to drive insertion on the one hand and its ability to dimerize on the other diminishes its usefulness in conveniently identifying amino acid sequences essential for dimerization. In contrast, our modified ''double reporter'' TOXCAT is capable of facile and simultaneous evaluation of the presence of intact protein, its membrane insertion, and the ability of its tm sequence to trigger oligomerization. Via random mutagenesis coupled with direct selection, this modified system also provides a means of rapidly identifying residues critical for mediating interactions between identical tm helices. Modified TOXCAT analysis of transmembrane segments of the human $Na_V1.5$ channel revealed the existence of two segments exhibiting apparent homodimerization when present within a single membrane spanning protein in the bacterial cell. Assessment of whether this ability of IS1 and IVS1 to dimerize is of physiologic importance in (e.g.) sodium channel clustering in cells clearly requires additional study.

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