A Functional Na\textsubscript{v}1.7-Na\textsubscript{v}Ab Chimera with a Reconstituted High-Affinity ProTx-II Binding Site

Ramkumar Rajamani, Sophie Wu, Ionyco Rodrigo, Mian Gao, Simon Low, Lisa Megson, David Wensel, Rick L. Pieschl, Debra J. Post-Munson, John Watson, David R. Langley, Michael K. Ahlijanian, Linda J. Bristow, and James Herrington


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ABSTRACT

The Na\textsubscript{v}1.7 voltage-gated sodium channel is implicated in human pain perception by genetics. Rare gain of function mutations in Na\textsubscript{v}1.7 lead to spontaneous pain in humans whereas loss of function mutations results in congenital insensitivity to pain. Hence, agents that specifically modulate the function of Na\textsubscript{v}1.7 have the potential to yield novel therapeutics to treat pain. The complexity of the channel and the challenges to generate recombinant cell lines with high Na\textsubscript{v}1.7 expression have led to a surrogate target strategy approach employing chimeras with the bacterial channel Na\textsubscript{v}Ab. In this report we describe the design, synthesis, purification, and characterization of a chimera containing part of the voltage sensor domain 2 (VSD2) of Na\textsubscript{v}1.7. Importantly, this chimera, DII S1–S4, forms functional sodium channels and is potently inhibited by the Na\textsubscript{v}1.7 VSD2 targeted peptide toxin ProTx-II. Further, we show by \textsuperscript{125}I ProTx-II binding and surface plasmon resonance that the purified DII S1–S4 protein retains high affinity ProTx-II binding in detergent. We employed the purified DII S1–S4 protein to create a scintillation proximity assay suitable for high-throughput screening. The creation of a Na\textsubscript{v}1.7-Na\textsubscript{v}Ab chimera with the VSD2 toxin binding site provides an important tool for the identification of novel Na\textsubscript{v}1.7 inhibitors and for structural studies to understand the toxin-channel interaction.

Introduction

The voltage-gated sodium channel Na\textsubscript{v}1.7, encoded by the gene SCN9A, has a compelling genetic link to human pain perception (Dib-Hajj et al., 2013). Rare mutations in SCN9A which result in overactive Na\textsubscript{v}1.7 channels cause autosomal dominant pain disorders such as inherited erythromelalgia and paroxysmal extreme pain disorder (reviewed by Dib-Hajj et al., 2010). In contrast, rare mutations that result in loss of function of Na\textsubscript{v}1.7 lead to the recessively inherited congenital insensitivity to pain (Cox et al., 2006, 2010; Goldberg et al., 2007). Reduced pain-related behaviors have also been observed in mice with global knockout of Na\textsubscript{v}1.7 or mice with selective knockout in specific sensory and/or sympathetic neurons (Gingras et al., 2014; Minett et al., 2012, 2014). These results are consistent with an important role for Na\textsubscript{v}1.7 in pain perception and suggest that selective inhibitors of Na\textsubscript{v}1.7 may have broad therapeutic potential in a variety of pain conditions.

Na\textsubscript{v}1.7, like all eukaryotic voltage-gated Na\textsubscript{v} channels, comprises four domains linked by cytoplasmic loops (Catterall, 2012). Each domain possesses a voltage sensor and pore module. Based on numerous studies over the last 60 years, Na\textsubscript{v} channels are expected to have a central pore formed from the S5–S6 segments and four voltage-sensing domains (VSD1–VSD4) each composed of the S1–S4 segments. Structural studies of bacterial Na\textsubscript{v} channels (Payandeh et al., 2011; Zhang et al., 2012) and the voltage-gated calcium channel Ca\textsubscript{v}1.1 (Wu et al., 2015; Wu et al., 2016) and the cockroach Na\textsubscript{v}PaS channel (Shen et al., 2017) support this general arrangement. The modular architecture has facilitated pharmacologic and structural studies of chimeric channels generated by the transfer of VSDs into related channels (Alabi et al., 2007; Bosmans et al., 2008; Ahuja et al., 2015; Klint et al., 2015).

The discovery of Na\textsubscript{v}1.7 inhibitors with sufficient selectivity across the Na\textsubscript{v} 1.X family to be effective pain therapeutic agents has proven to be a significant challenge. However, the identification of selective, small molecule aryl sulfonamide Na\textsubscript{v}1.7 inhibitors targeting the voltage sensor domain 4 (VSD4) have reinvigorated these efforts (McCormack et al., 2013; Focken et al., 2016). Furthermore, landmark structural studies of a chimeric channel where a portion of the VSD4 is grafted onto the bacterial Na\textsubscript{v} channel, Na\textsubscript{v}Ab, have provided key molecular insight into the determinants of selectivity (Ahuja et al., 2015). Despite the considerable promise of selective small-molecule inhibitors targeting Na\textsubscript{v}1.7 VSD4, confirmation of clinical efficacy has not yet been reported for these agents (Cao et al., 2016; Jones et al., 2016).
In addition to VSD4, several other regions of the Na\textsubscript{v}1.7 channel may be useful for targeting, including the outer pore (Walker et al., 2012; Thomas-Tran and Du Bois, 2016), inner vestibule (Bagnéris et al., 2014), and the other VSDs. Notably, voltage sensor domain 2 (VSD2) is the site of interaction of the selective peptide inhibitor ProTx-II (Schmalhofer et al., 2008) and related peptides (Park et al., 2014; Klint et al., 2015; Murray et al., 2015a,b, 2016). Also, this region has been targeted by antibody discovery efforts with some reported success (Lee et al., 2014). We sought to develop a surrogate target strategy through design of Na\textsubscript{v}1.7-Na\textsubscript{v}Ab chimeric channels incorporating the Na\textsubscript{v}1.7 ProTx-II binding site into the bacterial channel Na\textsubscript{v}Ab as a tool for new molecule discovery and structural studies. Here we report the functional characterization of a chimeric protein, referred to as DII S1–S4, and the development of screening assays using this reagent. The DII S1–S4 chimera will be useful in the search for novel Na\textsubscript{v}1.7 inhibitors targeting this region.

Materials and Methods

Na\textsubscript{v}Ab Chimera Construct Design

The design of the Na\textsubscript{v}Ab chimera construct focused on replacement of the extracellular loops and a significant segment of the transmembrane region of Na\textsubscript{v}Ab with the human DII Na\textsubscript{v}1.7 sequence. To identify regions of interest, sequence alignment (ClustalW with default settings) of Na\textsubscript{v}Ab against the human DII Na\textsubscript{v}1.7 domain II was performed to identify potential splice junctions within the VSD (Fig. 1). For the S1–S2 segment Na\textsubscript{v}1.7 (RefSeq NP_002968) the sequence range selected was ILE743-GLU799 covering the E1 loop region and VAL810-ARG830 for the SIII–SIV segment covering E2 loop based on conserved residues at the splice junctions. During the course of this effort, reports based on chimeric strategies to generate crystal structures (Abuja et al., 2015) and identify toxins (Klint et al., 2015) provided support to the viability of this approach.

Expression of Na\textsubscript{v}Ab Chimeras

Recombinant baculovirus (P1) were generated for BirA and all Na\textsubscript{v}Ab variants using the Bac-to-Bac system (Invitrogen, Carlsbad, CA). For large-scale expressions, high-titer baculovirus (P2) was generated by infecting S99 insect cells grown in Hyclone serum-free medium after the Bac-to-Bac protocol. S99 insect cells grown in ESF 921 medium (Expression Systems, Davis, CA) using a Wave Bioreactor System 20/50 EHT (GE Healthcare Life Sciences, Piscataway, NJ) was used for recombinant protein expression. S99 cells cultured to a density of 2.2 × 10^6 cells/ml were coinfectected with the P2 recombinant baculovirus (1–1.5 × 10^8 virus particles/ml) of Na\textsubscript{v}Ab chimera and BirA at a ratio of 2:1. The infected cells were maintained at 27°C for 65 hours with bioreactor wave settings suited for 8- to 10-liter volumes; the cell pellets were harvested by centrifugation. For expression of nonbiotinylated Na\textsubscript{v}Ab, the same protocol was used except that BirA was omitted.

ProTx-II Binding to Functional Na\textsubscript{v}1.7-Na\textsubscript{v}Ab Chimera

ProTx-II binding to Na\textsubscript{v}Ab chimeras or human Na\textsubscript{v}1.7 channels was determined with a [^{125}I]ProTx-II binding assay (Schmalhofer et al., 2008). For Na\textsubscript{v}Ab chimeras, S99 cells were collected 54 hours after transduction with baculovirus by centrifugation for 20 minutes at 2000 rpm. The cell pellets were washed once with PBS and stored at −70°C.

To prepare purified cell membranes, cell pellets were thawed, suspended in homogenization buffer (50 mM HEPES, 0.1% protease inhibitor cocktail [cat. no. P8340; Sigma-Aldrich], pH 7.4), and homogenized with 25 strokes of a glass Wheaton tissue grinder on ice. This homogenate was centrifuged at 500g for 10 minutes at 4°C on a Sorvall RC6plus centrifuge (Thermo Fisher Scientific, Waltham, MA). The supernatant was removed and centrifuged at 38,500g for 60 minutes at 4°C. This cell pellet was resuspended in assay buffer (50 mM HEPES, 130 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl\textsubscript{2}, pH 7.4), and the protein was determined with a Pierce BCA protein assay kit (Thermo Scientific Scientific) with bovine serum albumin (BSA) used as the protein standard.

ProTx-II Binding Assay

ProTx-II binding to functional Na\textsubscript{v}1.7-Na\textsubscript{v}Ab chimeras was performed in a 96 deep-well plate with a reaction volume of 100 µl. Non-specific binding was defined by cold ProTx-II (see the figure legends for concentrations). All radioligand incubations were performed at room temperature for 6 hours.

Cells and Reagents

The human embryonic kidney (HEK) 293 cell line stably expressing human Na\textsubscript{v}1.7 (5N/11S splice form) was purchased from Essen Biosciences (Ann Arbor, MI). ProTx-II and Huwetonin-IV-X was purchased from Peptides International (Louisville, KY). [^{125}I]ProTx-II was purchased from Perkin Elmer (Waltham, MA). N-terminal 8x His-tagged ProTx-II was a custom synthesis supplied by Smartox Biotechnology (Grenoble, France). Agitoxin-2 was purchased from Alomone Laboratories (Jerusalem, Israel).
For DII S1–S4 purified protein binding studies, the assay buffer contained 0.05% or 0.25% BSA. The binding reaction was terminated by filtration through GF/B filters for membranes and GF/F filters for purified protein, and the filters were washed 3 times with 2 ml of 4°C wash buffer (Dulbecco’s PBS with 10% fetal bovine serum for GF/B filters and 50 mM Tris HCl, pH 7.4, for GF/F filters). Filters were presoaked in 0.5% polyethylene amine. Bound radioactivity on the filters was counted on a Wallac Wizard gamma counter (Wallac/PerkinElmer, Gaithersburg, MD).

Specific [125I]ProTx-II binding from total binding for [125I]ProTx-II and expressed as mean counts per minute (cpm) ± S.E.M. IC50 values and saturation binding parameters were calculated from fits using GraphPad Prism 7 (GraphPad Software, San Diego, CA) as described in the figure legends. Statistical comparisons were performed using unpaired two-tailed t tests in Prism.

For whole-cell [125I]ProTx-II binding to human NaV1.7, HEK293 cells stably expressing human NaV1.7 were plated in 96-well poly-D-lysine-coated plates at 15,000 cells per well. After 4 days of culture, the medium was removed, and 100 μl of medium containing 0.3 nM [125I]ProTx-II was added. Nonspecific binding was determined by the inclusion of 600 nM unlabeled ProTx-II. [125I]ProTx-II was incubated with the cells for 6 hours at 37°C. At the end of the incubation, the radioligand was removed, and each well was washed twice with 200 μl of wash buffer (Dulbecco’s PBS, 10% fetal bovine serum). The cells were lysed by the addition of 200 μl of 0.2% SDS to each well. The contents of each well were pipetted into a 5-ml plastic tube and counted on a gamma counter.

For [125I]ProTx-II binding to human NaV1.7/HEK293 cell membranes, cells stably expressing human NaV1.7 channels were grown in T-175 flasks. The cells were detached using Detachin (Genlantis, San Diego, CA), pelleted by centrifugation for 10 minutes at 1000 rpm, and stored at –80°C. The human NaV1.7/HEK293 cell membranes were purified as described earlier for S9 membranes.

**Patch Clamp Electrophysiology**

Membrane currents were recorded using the whole-cell voltage clamp technique. Data were acquired at 50 kHz via a DPC interface with PatchMaster software (HEKA Instruments, Bellmore, NY). The glass microelectrodes had resistances of 1–4 MΩ when filled with intracellular solution. Series resistance was kept below 10 MΩ and compensated at least 50%. Leakage subtraction was performed with the P/4 method.

The methods used to record wild-type NaVAb and chimera channel currents in S9 cells were adapted from those described by Gamal El-Din et al. (2013). Briefly, S9 cells transduced with baculovirus were cryopreserved 24–31 hours after transduction. On the day of recording, the cells were rapidly thawed and plated in SF-900 II SFM (Thermo Fisher Scientific). The recordings were obtained between 0.5 hours and 5 hours after plating. The intracellular solution contained (in mM): 105 CsF, 35 NaCl, 10 EGTA, 10 HEPES, pH 7.4 with CsOH. The external solution contained (in mM): 140 NaCl, 2 MgCl2, 2 CaCl2, 10 HEPES, pH 7.4 with NaOH. The holding potential was −180 mV. ProTx-II II was diluted into the external solution supplemented with 0.1% BSA (w/v) and applied to cells by a gravity-fed bath perfusion system.

For recording NaV1.7 currents in HEK293 cells, the intracellular solution contained (in mM): 50 CsCl, 90 CsF, 10 NaF, 2 MgCl2, 10 EGTA, 10 HEPES, pH 7.2, with CsOH. The external solution was (in mM): 150 NaCl, 4 KCl, 1 MgCl2, 1.8 CaCl2, 10 HEPES, 10 glucose, pH 7.4 with NaOH. The holding potential was −110 mV. The peak membrane currents and tail current amplitudes were measured with PatchMaster software. Conductance was estimated by dividing the peak current at the test potential by the driving force for sodium. For the DII S1–S4 chimera, conductance was estimated by the tail current amplitude upon repolarization from the test potential. The voltage-dependence of activation was determined by fits of the Boltzmann equation of the form: G(V) = Gmax * (1/(1 + exp ((V – V1/2)/k))), where Gmax is the maximal conductance, V1/2 is the voltage of half-maximal activation, and k is the slope. Fitting and plotting of data were performed with Igor Pro 6 software (WaveMetrics, Lake Oswego, OR). Data are presented as mean ± S.E.M.

**SPP Assay**

Neuravidin (Pierce/Thermo Scientific, Rockford, IL) was amine-coupled to a Biacore T200 CM5 chip (GE Healthcare) via a standard EDC/NHS kit (GE Healthcare) using these parameters: 40 μg/ml Neuravidin in 10 mM sodium acetate, pH 4.5, at 37°C with 15 minutes contact time at 10 μl/min flow rate. These conditions resulted in the coupling of approximately 22,000 response units of Neuravidin to the chip surface. Neuravidin-coupled surfaces were conditioned with three pulses (30 seconds) of 1 M NaCl, 40 mM NaOH. Biotinylated NaVAb and chimeras were diluted to 100 nM in PBS with 0.1% w/v DDM and flowed over the Neuravidin surfaces at 25°C on separate flow cells until saturation (typically 1000 to 1200 response units). We used PBS + 0.1% DDM for all subsequent sample dilutions and running buffer, at a flow rate of 20 μl/min. Kinetic and steady-state affinity analyses of the resulting binding data were conducted with Biacore T200 Evaluation Software, version 2.0 (GE Healthcare).

[125I]ProTx-II Scintillation Proximity Assay

Scintillation proximity assays were performed in nontreated white 1536-well assay plates (Corning Life Sciences, Corning, NY). Briefly, 1.5 μl of assay buffer (130 mM NaCl, 5.4 mM KCl, 5 mM glucose, 0.8 mM MgCl2, 0.05% BSA, 0.1% DDM) was dispensed to the assay plate, followed by an equal volume of biotinylated DII S1–S4 protein (3.33 μg/ml). Streptavidin-coated polystyrene imaging beads (1.7 mg/ml; PerkinElmer) were then added, followed by 1.5 μl of radioligand (3.2 nM). Nonspecific binding was determined in the presence of 2 μM unlabeled ProTx-II. The final scintillation proximity assay (SPA) reaction (6 μl) contained 5 ng of DII S1–S4 protein, 2.5 μg/well of SPA imaging beads, and 0.8 nM [125I]ProTx-II. DMSO was included in the assay wells at a final concentration of 0.5%.

For competition binding experiments, unlabeled ProTx-II was diluted to 4 times the final concentration in assay buffer. The sample was diluted 1:3 in assay buffer in a 384-well polypropylene plate (Brooks Automation, Chelmsford, MA) to produce a 16-point dilution series. The final concentration of ProTx-II in the assay ranged from 2500 nM to 0.0017 nM.

Unlabeled peptide was preincubated with DII S1–S4 protein for 30 minutes before the addition of SPA beads and radioligand. After addition of SPA beads and radioligand, the plates were sealed with clear adhesive seals (PerkinElmer) and well luminescence was imaged immediately using a LEADseeker (instrument settings: 300-second exposure, 2 × 2 binning; Amersham, Pittsburgh, PA). The plates were then incubated at room temperature and imaged at 1-hour intervals for 12 hours.

Assay quality and robustness were estimated by calculating the Z’ statistic (Zhang et al., 1999) using the total binding wells (high signal) and nonspecific binding wells (low signal). Raw luminescence data were analyzed by nonlinear regression (four-parameter fit) using GraphPad Prism 7.

**Results**

**Design, Expression, and Characterization of NaV1.7-NaVAb Chimeras**

We aimed to design a NaV1.7-NaVAb chimeric protein that captured key elements of the NaV1.7 DII VSD (Fig. 1). Based on sequence alignment we selected a portion of the transmembrane segments including the extracellular loops to represent the target of interest (NaV1.7 DII VSD). The
in intracellular loops and the remaining segments of the transmembrane region of the template (NaVAb) were retained. Chimeric proteins were expressed in Sf9 cells using baculovirus techniques. Two independent constructs were generated (DII S1–S4 and DII S3–S4) for functional characterization and evaluation of suitability as a screening reagent (Supplemental Fig. 1).

**Electrophysiology of DII chimeras**

We explored whether the DII chimeras form functional channels when expressed in Sf9 cells. First, we confirmed that Sf9 cells expressing wild-type NaVAb displayed robust inactivating inward currents characteristic of NaVAb (Payandeh et al., 2012; Gamal El-Din et al., 2013) while no such currents were detectable in nontransduced Sf9 cells (Supplemental Fig. 2). Sf9 cells expressing DII S1–S4 chimera generated currents with distinct characteristics. In response to depolarizations above +50 mV, noninactivating outward currents were observed and upon repolarization to −180 mV large inward tail currents (Fig. 2A) were seen in 24 out of 39 cells. This current signature is consistent with a voltage-gated channel activating at positive membrane potentials. Tail current analysis estimated a reversal potential close to $E_{Na}$ (+37 mV, see Materials and Methods), consistent with a sodium conductance. The voltage-dependence of activation of DII S1–S4 channels estimated from tail current analysis suggested that activation is insignificant below 0 mV with a $V_{1/2}$ of +52 mV (Fig. 2B). This activation range is much more positive than wild-type NaVAb and wild-type human NaV1.7 (Fig. 2B; Supplemental Table 1). In contrast, DII S3–S4 chimera transduced Sf9 cells generated large, inactivating inward currents similar in appearance to NaVAb with a $V_{1/2}$ of −38 mV (Supplemental Fig. 3; Supplemental Table 1).

Next, DII S1–S4 chimera currents were tested for their sensitivity to ProTx-II. When measured at the test potential of +60 mV, 10 nM ProTx-II inhibited the DII S1–S4 tail current by $94\% \pm 2\%$ ($n = 3$). Inhibition was characterized by a positive shift in the voltage-dependence of activation (Fig. 2D). To quantify the effect of ProTx-II on DII S1–S4 activation we chose a concentration of 100 nM, to avoid long incubation times because of the relative instability of these recordings. At 100 nM, ProTx-II shifted activation by +54 mV and decreased maximal conductance by 24% (Supplemental Fig. 4). Hence, ProTx-II is a potent inhibitor of DII S1–S4 channels and acts as a gating modifier, similar to the action of ProTx-II on NaV1.7. In contrast, DII S3–S4 channels were not significantly inhibited by 100 nM ProTx-II ($5\% \pm 11\%$ $n = 6$; data not shown).

**Purification of NaV1.7-NaVAb Chimeras**

Once expression and function were confirmed, large-scale cell cultures (8–10 liters) were transduced to yield cell pellets for membrane isolation and protein purification. Wild-type NaVAb and chimeras were purified on anti-Flag M2 affinity columns, and eluted protein was solubilized in detergent (0.1% w/v) DDM. SDS-PAGE analysis demonstrated proteins with the predicted molecular mass (22 kDa; Supplemental Fig. 5). Protein purity was estimated to be greater than 90%.

**[125I]ProTx-II Binding**

Radiolabeled ProTx-II binding was also used to test for ProTx-II sensitivity of DII chimeras. A baseline was established with ProTx-II binding to human NaV1.7. As expected, HEK293 cells stably expressing wild-type human NaV1.7 channels displayed robust specific binding of [125I]ProTx-II (Fig. 3A). In membrane preparations from these same cells, no specific binding of [125I]ProTx-II was detectable. The average specific binding window in cells was 5.6-fold compared with 1.1-fold in membranes ($P < 0.05$).

In membrane preparations from Sf9 cells expressing the DII S1–S4 chimera, significant specific binding of [125I]ProTx-II was apparent (Fig. 3B). Interestingly, nontransduced Sf9 cells displayed a lower but measurable total [125I]ProTx-II binding, which was similar to that observed in cells expressing either [125I]ProTx-II. (A) Representative recording from a Sf9 cell transduced with BacMam encoding DII S1–S4 chimera. Currents were evoked by 20-millisecond step depolarizations between −140 mV and +120 mV. For clarity, only the step depolarizations between −20 mV and +100 mV in 20 mV increments are shown. (B) Plot of the mean ± S.E.M. relative conductance of NaVAb ($\bigcirc$, $n = 9$), human NaV1.7 ($\triangle$, $n = 12$), and DII S1–S4 chimera ($\blacksquare$, $n = 9$) versus membrane potential. The solid lines are fits of the Boltzmann equation to the data. Parameters of the fits are given in Supplemental Table 1. (C) Representative recording from a Sf9 cell transduced with BacMam encoding DII S1–S4 chimera. Currents were evoked by 20-millisecond step depolarization to +80 mV in control and in the presence of 10 nM ProTx-II. (D) Plot of the relative conductance versus membrane potential for the recording in panel C in control ($\bigcirc$) and 100 nM ProTx-II ($\blacktriangleright$). Conductance was estimated from the tail current amplitude and normalized to the control amplitude at the +120 mV test potential.
wild-type NaVAb or the DII S3–S4 chimera (Fig. 3B). In all cases this binding was largely displaced by cold ProTx-II, suggesting potential alternate site(s) of specific interaction on the S9 membrane.

Nevertheless, the highest specific [125I]ProTx-II binding was observed in S9 membranes expressing the DII S1–S4 chimera (7.2-fold compared with about 4-fold for S9, NavAb, or DII S3–S4; \( P < 0.05 \)), suggesting direct binding to the DII S1–S4 chimeric protein. Given this observation, we purified DII S1–S4 protein and tested for [125I]ProTx-II binding. Specific [125I]ProTx-II binding was observed with purified DII S1–S4 protein in detergent with a clear dependence on the amount of chimeric protein (Fig. 3C). The highest specific binding was observed at 0.3 \( \mu \)g protein (8.8-fold, \( P < 0.05 \) compared with the other protein concentrations). No specific [125I]ProTx-II binding was detectable with purified DII S3–S4 protein (Supplemental Fig. 6).

The [125I]ProTx-II binding data with S9 membranes and purified proteins suggested that the DII S1–S4 chimera but not the DII S3–S4 chimera reconstituted the high-affinity ProTx-II binding site. To characterize this binding further, we performed competition binding experiments on purified DII S1–S4 protein with cold ProTx-II and a related peptide, Huwentoxin-IV (Fig. 3D). Cold ProTx-II displaced specific [125I]ProTx-II binding with an IC50 of 36 nM whereas Huwentoxin-IV was weaker (IC50 of 662 nM), consistent with the literature (Xiao et al., 2010). Saturation binding experiments of [125I]ProTx-II binding to the DII S1–S4 chimera estimated an equilibrium dissociation constant (\( K_d \)) of 9.4 nM (Supplemental Fig. 7).

### SPR Characterization

To explore the kinetics of binding interactions between the toxins and DII chimeras, biotinylated versions of the purified NavAb, DII S3–S4, and DII S1–S4 proteins were captured onto a Biacore CM5 chip derivatized with Neutravidin. His-tagged ProTx-II, Huwentoxin-IV, and Agitoxin-2 were then flowed over these surfaces to detect binding interactions. As shown in Fig. 4, ProTx-II and Huwentoxin-IV bound specifically to the DII S1–S4 chimera but not to NaVAb or DII S3–S4 chimera. Agitoxin-2 did not bind to any of these surfaces, as expected (data not shown). To test the reproducibility of this system, concentration series of the ProTx-II and Huwentoxin-IV were flowed over these surfaces in duplicate. As shown in Fig. 4, C and D, concentration-dependent binding was detected for both toxins, and sample duplicates demonstrated good reproducibility of the binding signal.

These binding data did not fit well to a 1:1 binding model, so on- and off-rates could not be accurately determined. Fitting with a steady-state binding model yielded apparent affinities of 15 nM for ProTx-II and 125 nM for Huwentoxin-IV, consistent with the rank order reported in literature for wild-type human NaV1.7 (Xiao et al., 2010).

### SPA Assay

Given the robust binding of ProTx-II to purified DII S1–S4 protein detected by filter binding and SPR, we next sought to develop a SPA for screening. Biotinylated DII S1–S4 protein was immobilized on streptavidin-coated SPA beads and incubated with [125I]ProTx-II. Initial studies were performed in 384-well format, and subsequently the assay was optimized for 1536-well plates.
Optimization for protein and $^{[125]}$IProTx-II usage yielded an assay with suitable sensitivity to competition by cold ProTx-II (IC$_{50}$ = 107 nM; Fig. 5A). The IC$_{50}$ of competition by cold ProTx-II peptide was stable for at least 8 hours (average IC$_{50}$: 118 ± 12 nM; range: 99–135 nM) (Fig. 5B). The reduced potency of ProTx-II in this assay compared with filter binding was likely due to ligand depletion in the low volume format (6 μl).

The signal-to-background ratio increased slightly over the first hour of incubation, then was stable for 10 hours at room temperature (average: 7.0 ± 0.43), and decreased to the approximate $t = 0$ level by 12 hours (Fig. 5C). Assay robustness, as indicated by the Z’ statistic, was stable over the course of the experiment (average $Z’$: 0.62 ± 0.03). As expected, the assay signal was also sensitive to Huwentoxin-IV with reduced potency (IC$_{50}$ = 1.3 μM) compared with ProTx-II (data not shown).

**Discussion**

NaV1.7 is a target of high importance for the development of novel analgesics based on human genetics linking this channel to pain perception. Two sites on NaV1.7 are known to yield selective inhibitors of channel function. Small molecule sulfonamides bind to VSD4 and lock the VSD in the activated conformation, leading to channel inactivation (McCormack et al., 2013; Ahuja et al., 2015). The other site is on VSD2, where gating modifier peptides such as ProTx-II bind (Schmalhofer et al., 2008). ProTx-II inhibits Na$_V$ channels by a distinctly different mechanism—it traps the voltage sensor in the resting state, making it more difficult for voltage to open the channel (Sokolov et al., 2008).

Although both sites are of significant interest, it is not yet known if these mechanistically distinct approaches to channel inhibition provide any advantage from a therapeutic standpoint. Nevertheless, efforts to thoroughly explore each of these pharmacophores have been rigorous. Here we describe the characterization of a novel chimeric protein comprising the human NaV1.7 DII S1–S4 region with the bacterial NaVAb, which is suitable for high-throughput screening for novel agents targeting the VSD2 site.

We found the DII S3–S4 and DII S1–S4 chimeras form functional channels with unique properties. Most notably, in comparison with NaVAb, there is a positive shift in activation of...
55 mV for DII S3–S4 and 145 mV for DII S1–S4. The DII S3–S4 represents an activation profile closer to that observed for wild-type NaVAb, but the DII S1–S4 profile renders it such that channel opening is negligible at 0 mV. This activation profile uniquely qualifies the DII S1–S4 for use in identification of modulators that target primarily target the resting state of the channel.

We speculate that the large shift in activation profiles originates from changes in the pairing environment of the gating charges spanning the S4 segment of the DII VSDs and can vary depending on the template used. A right shift in the activation potential for the Kv2.1-rat DII NaV1.2 chimera compared with the template reference (Kv2.1) was also observed in previously reported studies (Bosmans et al., 2008).

Previous studies have found that transferring a section of the helix-turn-helix motif covering the E3 loop of NaV1.7 DII S3–S4 to Kv2.1 conferred ProTx-II sensitivity to the channel chimera (Bosmans et al., 2008). Additionally, mutation of individual residues in the DII S3–S4 region (e.g., F813 and E818) reduces ProTx-II sensitivity (Schmalhofer et al., 2008; Xiao et al., 2010). Likewise, a residue (L833) in DII S3 of NaV1.2 is important for ProTx-II inhibition (Sokolov et al., 2008).

In contrast, mutation of residues in DII S1–S2 of NaV1.5 did not significantly affect ProTx-II inhibition (Smith et al., 2007). We find that transfer of NaV1.7 DII S3–S4 helix-turn-helix motif to NaVAb did not yield channels sensitive to ProTx-II. One possible reason for the difference in observation could have been a result of the variation in the inserted sequence. To reconstitute high-affinity ProTx-II binding, the helix-turn-helix motif of NaV1.7 DII S1–S2 was also required. These results suggest that in the context of NaVAb template additional residues of NaV1.7 DII S1–S2 are likely important for ProTx-II binding.

Gating modifier peptides stabilize the resting conformation, and affinity is reduced when the voltage sensor is in the activated conformation (Tilley et al., 2014). Hence, the positive activation profile may have allowed for detection of high-affinity ProTx-II binding in membranes and with purified protein. Despite the high-affinity binding observed with the purified DII S1–S4 chimera ($K_d = 30 \text{ nM}$), the binding is weaker than that observed with wild-type human NaV1.7 expressed in HEK293 cells ($K_d = 0.3 \text{ nM}$) (Schmalhofer et al., 2008). The lower binding affinity observed with DII S1–S4 may result from a combination of alternate VSD conformations and/or the influence of the detergent, resulting in significantly different environment compared with live HEK293 cells expressing wild-type NaV1.7 (Marheineke et al., 1998). Gating modifier peptides are known to partially partition into membranes (Lee and MacKinnon, 2004; Milescu et al., 2007; Mihailescu et al., 2014), and ProTx-II clearly interacts with phospholipids (Henriques et al., 2016). Conversely, VSD interactions with lipid can influence toxin binding (Smith et al., 2005; Milescu et al., 2009). Auxiliary subunits are also known to influence gating modifier action on NaV channels (Wilson et al., 2011). Thus, the affinity of DII S1–S4 for ProTx-II is likely impacted by the expression/reconstitution system employed here. Alternatively, other residues present in NaV1.7 VSD2 but not included in the DII S1–S4 chimera may be important for ProTx-II binding either through direct interaction or providing key structural features of the VSD. If so, further refinements of the DII S1–S4 chimera may improve ProTx-II binding affinity. Also, it is important to note that the other VSDs of NaV1.7 appear to interact with ProTx-II (Bosmans et al., 2008; Xiao et al., 2010, 2014), which would not be captured in the chimera reported here.

Purified DII S1–S4 protein in detergent capable of high-affinity ProTx-II binding opens several possibilities for use in discovery efforts. For example, the protein may be useful as an antigen in monoclonal antibody discovery, screening reagents to identify molecules such as toxins, adnectins, synthetic peptides, “milla” molecules, and small molecules. Screening approaches based on detection of binding to the chimera (and not wild-type NaVAb) may yield molecules that interact specifically with the transplanted residues of DII S1–S4. Furthermore, traditional screening approaches based on competition of $^{125}$I-ProTx-II binding are feasible.

The ability of purified DII S1–S4 to bind ProTx-II affords several opportunities for assay development not possible with crude membrane preparations containing channels at relatively low density. For example, we were able convert and miniaturize the $^{125}$I-ProTx-II binding assay to an SPA format in 1536 wells. The homogenous assay is sufficiently robust to support high-throughput screening, with a large window that is stable over several hours. We note that the observed IC$_{50}$ of cold ProTx-II peptide in the SPA assay is approximately 5-fold greater than that observed in filter binding experiments. The final SPA conditions required 5 ng of chimeric channel to yield an acceptable assay, which corresponds to a 27-fold molar excess of chimeric protein over the radioligand.

The lower potency of cold ProTx-II in the SPA format may be the result of ligand depletion. The effect of ligand depletion on the measured IC$_{50}$ ligand depletion as has been documented for other SPAs (Carter et al., 2007). SPAs for other ion channels have been based on membrane preparations rather than purified protein (Hui et al., 2005; Zhang et al., 2006). One precaution common to any chimera approach is the need to test any hits identified from a screen for activity against the wild-type NaV1.7 in a functional assay.

SPR is a powerful method to study protein-peptide interactions in detail. Alpha-scorpion toxin binding to isolated NaV1.2 VSD4 can be detected by SPR (Martin-Eauclaire et al., 2015). Here we show that an SPR assay-based immobilization of an entire chimeric channel (DII S1–S4) is sensitive to ProTx-II binding. No binding was detectable with immobilized wild-type NaVAb or the DII S3–S4 chimera, confirming that the SPR signal reflects a specific interaction with the NaV1.7 DII chimera. Furthermore, the estimated $K_d$ based on SPR aligns well with $^{125}$I-ProTx-II binding competition and electrophysiology studies.

A robust SPR assay for the DII S1–S4 chimera has several potential uses. For example, such an assay is ideal for confirming direct, specific interaction of novel molecules identified by screening with the chimera. Also, monoclonal antibodies identified from either traditional hybridoma approaches or phage panning can be tested for binding. Another obvious use of such an assay is the biophysical characterization of the interaction of molecules with the chimeric channel, including kinetic and affinity estimates.

In summary, here we report a functional NaV1.7 DII chimera with the bacterial channel NaVAb. The chimera forms functional channels and is sensitive to the NaV1.7 gating modifier peptide ProTx-II. We demonstrate the utility of this chimera by engineering both an SPA and SPR assay with purified protein. The DII S1–S4 chimera will prove useful for the discovery and characterization of novel molecules targeting this important functional domain of NaV1.7. Furthermore, this chimera approach may have broad applicability.
for enabling screening at other membrane protein targets of pharmacoeconomic interest.

Authorship Contributions

Participated in research design: Rajamani, Gao, Watson, Langley, Ahlijanian, Bristow, Herrington.

Conducted experiments: Wu, Rodrigo, Low, Megson, Wensel, Pieschl, Post-Munson, Watson, Herrington.

Contributed new reagents or analytical tools: Gao, Megson.

Performed data analysis: Rodrigo, Low, Megson, Wensel, Pieschl, Post-Munson, Watson, Herrington.

Wrote or contributed to the writing of the manuscript: Rajamani, Wu, Gao, Wensel, Pieschl, Ahlijanian, Bristow, Herrington.

References


Address correspondence to: Dr. Ramkumar Rajamani, Bristol-Myers Squibb, 5 Research Parkway, Wallingford, CT 06492. E-mail: ramkumar.rajaman@bms.com
A functional Na\textsubscript{v}1.7- Na\textsubscript{v}Ab chimera with a reconstituted high affinity ProTx-II binding site

Ramkumar Rajamani, Sophie Wu, Iyoney Rodrigo, Mian Gao, Simon Low, Lisa Megson, David Wensel, Rick L. Pieschl, Debra J. Post-Munson, John Watson, David R. Langley, Michael K. Ahlijanian, Linda J. Bristow, and James Herrington

Address for correspondence:
Ramkumar Rajamani, Ph.D.
Bristol-Myers Squibb
5 Research Parkway
Wallingford, CT 06492
Ramkumar.Rajamani@bms.com
Supplemental Figure 1. Chimeric NavAb-DII-S1-S4 and NavAb-DII-S3-S4 amino acid sequence of channels expressed in Sf9 cells.

**NavAb-DII-S1-S4**

MDYKDDDDKGSVLVRGSMLRITNIVESSFTTKFITICTIVLNTLFMAMEHHMTEEFKNVLAIGNLVFTGFAAEIILRIYVHRISFFKDPWSLFDFVFVTLSVELFLADVEGLSVLRSFRLLRLFRLVTAVPQMRKVSLISVIPGMLSVIALM TLFFYIFAIMATQLFGERFPEWFGTGLGESFYTLFQQMTLESWSMGIVRPLMEVYPYAWVFFIFFIFVVTFVMINLVVAILNDAMAILNQKEEQHIIDEVQSHEDVINNEIIKLREEEIVELKELIKTSKLN

**NavAb-DII-S3-S4**

MDYKDDDDKGSVLVRGSMLRITNIVESSFTTKFIIYLIVLNGITMGLETSTKTFMQVAYTTLFNQIVITIFTIEIILRIYVHRISFFKDPWSLFDFVFVTLSVELFLADVGLSVLRSFRLLRLFRLVTAVPQMRKVSLISVIPGMLSVIALMTLFFYIFAIMATQLFGERFPEWFGTGLGESFYTLFQQMTLESWSMGIVRPLMEVYPYAWVFFIFFIFVVTFVMINLVVAILNDAMAILNQKEEQHIIDEVQSHEDVINNEIIKLREEEIVELKELIKTSKLN
Supplemental Figure 2. Functional expression of NavAb channels in Sf9 cells. A. Representative recording from a non-transduced Sf9 cell. Currents in response to 20 ms step depolarizations between -120 mV and +120 mV in 10 mV increments are shown. B. Representative recording from a Sf9 cell transduced with BacMam encoding wild-type NavAb. Currents were evoked by 20 ms step depolarizations between -140 mV and 0 mV. For clarity, step depolarizations in 20 mV increments are shown.
Supplemental Figure 3. Functional expression of DII S3-S4 chimera in Sf9 cells. A. Representative recording from a Sf9 cell transduced with BacMam encoding DII S3-S4 chimera. Currents in response to 20 ms step depolarizations between -140 mV and 0 mV in 10 mV increments are shown. B. Plot of mean relative conductance (± S.E.M.) versus step potential for DII S3-S4 chimera channels estimated from tail current analysis. Tail current amplitudes were normalized to the amplitude following the step to 0 mV for each cell (n=7). The solid line is a fit of the Boltzmann equation to the data. Parameters of the fit are given in Table 1.
Supplemental Figure 4. Effect of ProTx-II on the voltage-dependent gating of DII S1-S4 chimera channels. Plot of mean relative conductance (± S.E.M.) versus step potential for DII S3-S4 chimera channels in control (circles) and following application of 100 nM ProTx-II (squares). Conductance was estimated from peak current measurements and normalized to the conductance following the control step to +120 mV for each cell (n=7). The solid line is a fit of the Boltzmann equation to the data. Parameters of the fit in Control are: $V_{1/2} = 52.7$ mV, slope = 15.1 mV, Maximum conductance = 1.0. For the fit in 100 nM ProTx-II, the parameters are: $V_{1/2} = 106.8$ mV, slope = 14.8 mV, maximum conductance = 0.76.
Supplemental Figure 5. Purified NavAb DII S1-S4 chimera. Representative SDS-PAGE gel of the final purified material stained with Instant Blue. Lanes 1, 3 and 5 were loaded with 2 µg, 4 µg or 8 µg protein, respectively. MW markers were loaded in lanes 2 and 4.
Supplemental Figure 6. Specific binding of $[^{125}\text{I}]\text{ProTx-II}$ to purified DII S1-S4 chimera but not DII S3-S4 chimera. Biotinylated DII S3-S4 or DII S1-S4 protein (0.3 µg) in DDM were incubated with 1.67 nM $[^{125}\text{I}]\text{ProTx-II}$. To define non-specific (NS) binding, cold ProTx-II (2 µM) was included in the binding reaction. Mean cpm ± S.E.M. (n=3) are plotted.
Supplemental Figure 7. Saturation binding of $^{[125\text{I}]}$ProTx-II to purified DII S1-S4 chimera. DII S1-S4 protein was incubated with 0.2-10.5 nM $^{[125\text{I}]}$ProTx-II and specific binding determined. Mean bound $^{[125\text{I}]}$ProTx-II bound ± S.E.M. is plotted versus free $^{[125\text{I}]}$ProTx-II concentration. Each point represents the mean of five experiments measured in triplicate. The solid line is a fit of a single site model to the data with the parameters $B_{\text{max}} = 309$ nmol/mg, $K_d = 9.8$ nM.
Supplemental Table 1. Activation parameters of wild-type and chimeric channels. Values are derived from the fits of the Boltzmann equation to the normalized mean conductance versus membrane potential. For wt NavAb, wt hNav1.7 and DII S3-S4 chimera, conductance was estimated from peak current measurements. For the DII S1-S4 chimera, tail current amplitudes were used to estimate conductance.

<table>
<thead>
<tr>
<th>Channel</th>
<th>$V_{1/2}$ (mV)</th>
<th>slope (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt NavAb</td>
<td>-93.4</td>
<td>7.7</td>
</tr>
<tr>
<td>wt hNav1.7</td>
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<td>6.9</td>
</tr>
<tr>
<td>DII S1-S4</td>
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</tr>
<tr>
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<td>10.2</td>
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