A Functional Na_v1.7-Na_vAb Chimera with a Reconstituted High-Affinity ProTx-II Binding Site[§]

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

Molecular Discovery Technologies, Wallingford, Connecticut, Princeton, New Jersey, and Waltham, Massachusetts (R.R., S.W., I.R., M.G., S.L., L.M., D.W., D.R.L.); Discovery Biology (R.L.P., D.J.P.-M., M.K.A., L.J.B., J.H.) and Lead Discovery and Optimization (J.W.), Bristol-Myers Squibb Company, Wallingford, Connecticut

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ABSTRACT

The Na_v1.7 voltage-gated sodium channel is implicated in human pain perception by genetics. Rare gain of function mutations in Na_v1.7 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_v1.7 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_v1.7 expression have led to a surrogate target strategy approach employing chimeras with the bacterial channel Na_vAb. 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_v1.7. Importantly, this chimera, DII S1–S4, forms functional sodium channels and is potently inhibited by the Na_v1.7 VSD2 targeted peptide toxin ProTx-II. Further, we show by [¹²⁵I]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_v1.7-Na_vAb chimera with the VSD2 toxin binding site provides an important tool for the identification of novel Na_v1.7 inhibitors and for structural studies to understand the toxin-channel interaction.

Introduction

The voltage-gated sodium channel Na_v1.7, 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_v1.7 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_v1.7 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_v1.7 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_v1.7 in pain perception and suggest that selective inhibitors of Na_v1.7 may have broad therapeutic potential in a variety of pain conditions.

Na_v1.7, like all eukaryotic voltage-gated Na_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_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_v channels (Payandeh et al., 2011; Zhang et al., 2012) and the voltage-gated calcium channel Ca_v1.1 (Wu et al., 2015; Wu et al., 2016) and the cockroach Na_vPaS 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_v1.7 inhibitors with sufficient selectivity across the Na_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_v1.7 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_v channel, Na_vAb, 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_v1.7 VSD4, confirmation of clinical efficacy has not yet been reported for these agents (Cao et al., 2016; Jones et al., 2016).

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ABBREVIATIONS: BSA, bovine serum albumin; DDM, *n*-dodecyl- β -D-maltoside; HEK, human embryonic kidney cells; *K*_d, equilibrium dissociation constant; Na_v, voltage-gated sodium channel; PBS, phosphate-buffered saline; SPA, scintillation proximity assay; SPR, surface plasmon resonance; VSD, voltage sensing domain; VSD2, voltage sensor domain 2.

In addition to VSD4, several other regions of the Na_v1.7 channel may be useful for targeting, including the outer pore (Walker et al., 2012; Thomas-Tran and Du Bois, 2016), inner vestibule (Bagnris 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_v1.7-Na_vAb chimeric channels incorporating the Na_v1.7 ProTx-II binding site into the bacterial channel Na_vAb 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_v1.7 inhibitors targeting this region.

Materials and Methods

Na_vAb Chimera Construct Design

The design of the Na_vAb chimera construct focused on replacement of the extracellular loops and a significant segment of the transmembrane region of Na_vAb with the human DII Na_v1.7 sequence. To identify regions of interest, sequence alignment (ClustalW with default settings) of Na_vAb against Na_v1.7 domain II was performed to identify potential splice junctions within the VSD (Fig. 1). For the S1–S2 segment Na_v1.7 (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 (Ahuja et al., 2015) and identify toxins (Klint et al., 2015) provided support to the viability of this approach.

Expression of Na_vAb Chimeras

Recombinant baculovirus (P1) were generated for BirA and all Na_vAb variants using the Bac-to-Bac system (Invitrogen, Carlsbad, CA). For large-scale expressions, high-titer baculovirus (P2) was generated by infecting Sf9 insect cells grown in Hyclone serum-free medium after the Bac-to-Bac protocol. Sf9 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. Sf9 cells cultured to a density of 2.2×10^6 cells/ml were coinfecting with the P2 recombinant baculovirus ($1\text{--}1.5 \times 10^8$ virus particles/ml) of Na_vAb 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_vAb, the same protocol was used except that BirA was omitted.

Protein Purification

The Sf9 cell pellet was resuspended and homogenized in a buffer containing 50 mM Tris (pH 8.0), 250 mM NaCl, benzonase (Sigma-Aldrich, St. Louis, MO), and protease inhibitor cocktail (Sigma-Aldrich). Cells were then lysed by sonication. The total cell lysate was incubated with 1% (w/v) *n*-dodecyl- β -D-maltoside (DDM; Anatrace, Maumee, OH) at 4°C for 1 hour. Cell debris was removed by centrifugation, and the supernatant was collected and incubated with anti-Flag M2 affinity resin (Sigma-Aldrich) at 4°C for overnight for binding.

The protein was eluted with a buffer containing 25 mM Tris (pH 8.0), 250 mM NaCl, 0.1% (w/v) DDM, and $100 \mu\text{g ml}^{-1}$ 3x Flag peptide (Sigma-Aldrich). The eluted protein was concentrated and loaded onto Superdex-200 columns (GE Healthcare) in Dulbecco's phosphate-buffered saline (PBS) buffer containing 0.1% (w/v) DDM. The peak fraction was collected for all the subsequent work.

The majority of the chimera constructs behaved similarly during purification. The level of biotinylation was assessed by liquid chromatography with mass spectrometry and estimated to be at least 50% of the total.

Cells and Reagents

The human embryonic kidney (HEK) 293 cell line stably expressing human Na_v1.7 (5N/11S splice form) was purchased from Essen Biosciences (Ann Arbor, MI). ProTx-II and Huwentoxin-IV were purchased from Peptides International (Louisville, KY). [¹²⁵I]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).

[¹²⁵I]ProTx-II Binding Assay

ProTx-II binding to Na_vAb chimeras or human Na_v1.7 channels was determined with a [¹²⁵I]ProTx-II binding assay (Schmalhofer et al., 2008). For Na_vAb chimeras, Sf9 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, 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.

Binding experiments were performed in a 96 deep-well plate with a reaction volume of 100 μl . Nonspecific binding was defined by cold ProTx-II (see the figure legends for concentrations). All radioligand incubations were performed at room temperature for 6 hours.

Fig. 1. Design of Na_v1.7-Na_vAb chimeras. Sequence alignment of the S1–S2 (top) and S3–S4 (bottom) regions of Na_vAb with human Na_v1.7 domain II. The splice junctions chosen for creation of chimeras are designated by the anchor symbols.

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 [125 I]ProTx-II binding values were calculated by subtraction of nonspecific binding from total binding for [125 I]ProTx-II and expressed as mean counts per minute (cpm) $\pm$ 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 [125 I]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 μ l of medium containing 0.3 nM [125 I]ProTx-II was added. Nonspecific binding was determined by the inclusion of 600 nM unlabeled ProTx-II. [125 I]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 μ l of wash buffer (Dulbecco's PBS, 10% fetal bovine serum). The cells were lysed by the addition of 200 μ l 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 [125 I]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 was described earlier for Sf9 membranes.

Patch Clamp Electrophysiology

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

The methods used to record wild-type NavAb and chimera channel currents in Sf9 cells were adapted from those described by Gamal El-Din et al. (2013). Briefly, Sf9 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 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: $G(V) = G_{\text{max}} * (1/(1 + \exp(-(V - V_{1/2})/k)))$, where G_{max} is the maximal conductance, $V_{1/2}$ 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 $\pm$ S.E.M.

SPR Assay

Neutravidin (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 μ g/ml Neutravidin in 10 mM sodium acetate, pH 4.5, at 37°C with 15 minutes contact time at 10 μ l/min flow rate. These conditions resulted in the coupling of approximately 22,000 response units of Neutravidin to the chip surface. Neutravidin-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 Neutravidin 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 μ l/min. Kinetic and steady-state affinity analyses of the resulting binding data were conducted with Biacore T200 Evaluation Software, version 2.0 (GE Healthcare).

[125 I]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 μ l 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 μ g/ml). Streptavidin-coated polystyrene imaging beads (1.7 mg/ml; PerkinElmer) were then added, followed by 1.5 μ l of radioligand (3.2 nM). Nonspecific binding was determined in the presence of 2 μ M unlabeled ProTx-II. The final scintillation proximity assay (SPA) reaction (6 μ l) contained 5 ng of DII S1–S4 protein, 2.5 μ g/well of SPA imaging beads, and 0.8 nM [125 I]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

intracellular loops and the remaining segments of the transmembrane region of the template (Na_vAb) 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 Na_vAb displayed robust inactivating inward currents characteristic of Na_vAb (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 Na_vAb and wild-type human Na_v1.7 (Fig. 2B; Supplemental Table 1). In contrast, DII S3–S4 chimera transduced Sf9 cells generated large, inactivating inward currents similar in appearance to Na_vAb with a $V_{1/2}$ of activation 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 Na_v1.7. 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 Na_v1.7-Na_vAb 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 Na_vAb 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 Na_v1.7. As expected, HEK293 cells stably expressing wild-type human Na_v1.7 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

Fig. 2. DII S1–S4 chimera forms functional ProTx-II sensitive channels. (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 $\pm$ S.E.M. relative conductance of Na_vAb (●, $n = 9$), human Na_v1.7 (○, $n = 12$), and DII S1–S4 chimera (■, $n = 9$) 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 a 20-millisecond step depolarization to +60 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 (●) and 100 nM ProTx-II (▲). Conductance was estimated from the tail current amplitude and normalized to the control amplitude at the +120 mV test potential.

Fig. 3. $[^{125}\text{I}]\text{ProTx-II}$ binding of Nav1.7-NavAb chimeras. (A) $[^{125}\text{I}]\text{ProTx-II}$ binding to human Nav1.7 channels expressed in HEK293 cells. For whole-cell binding (left), 15,000 cells per well in a 96-well plate were incubated with 0.36 nM $[^{125}\text{I}]\text{ProTx-II}$. Nonspecific (NS) binding was defined by the addition of 0.6 μM cold ProTx-II . Bars represent the mean $\pm$ S.E.M. cpm, $n = 6$. For membranes (right), 50 μg of human Nav1.7 HEK membranes were incubated with 0.15 nM $[^{125}\text{I}]\text{ProTx-II}$. Nonspecific binding was defined by the addition of 1 μM cold ProTx-II . Bars represent the mean $\pm$ S.E.M. cpm, $n = 3$. (B) We incubated 1 μg Sf9 membranes from nontransduced cells (Sf9) or cells transduced with either wild-type NavAb , DII S3–S4 chimera, or DII S1–4 chimera with 1.2 nM $[^{125}\text{I}]\text{ProTx-II}$. Nonspecific binding was defined by addition of 20 μM cold ProTx-II . Bars represent the mean $\pm$ S.E.M. cpm, $n = 3$. (C) Purified DII S1–S4 protein (0.003–3.0 μg) was incubated with 1.9 nM $[^{125}\text{I}]\text{ProTx-II}$. Nonspecific binding was defined by the addition of 0.6 μM cold ProTx-II . Bars represent the mean $\pm$ S.E.M. cpm, $n = 3$. (D) Percentage of inhibition of specific $[^{125}\text{I}]\text{ProTx-II}$ (1.1 nM) binding to purified DII S1–S4 protein (0.3 μg) by cold ProTx-II (●) and Huwentoxin-IV (□). Nonspecific binding was defined by the addition of 2 μM cold ProTx-II . Symbols represent the mean $\pm$ S.E.M. inhibition of three independent experiments measured in triplicate. The estimated IC_{50} from the fits is 36 nM (ProTx-II) and 662 nM (Huwentoxin-IV).

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 Sf9 membrane.

Nevertheless, the highest specific $[^{125}\text{I}]\text{ProTx-II}$ binding was observed in Sf9 membranes expressing the DII S1–S4 chimera (7.2-fold compared with about 4-fold for Sf9, 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 $[^{125}\text{I}]\text{ProTx-II}$ binding. Specific $[^{125}\text{I}]\text{ProTx-II}$ 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 μg protein (8.8-fold, $P < 0.05$ compared with the other protein concentrations). No specific $[^{125}\text{I}]\text{ProTx-II}$ binding was detectable with purified DII S3–S4 protein (Supplemental Fig. 6).

The $[^{125}\text{I}]\text{ProTx-II}$ binding data with Sf9 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 $[^{125}\text{I}]\text{ProTx-II}$ binding with an IC_{50} of 36 nM whereas Huwentoxin-IV was weaker (IC_{50} of 662 nM), consistent with the literature (Xiao et al., 2010). Saturation binding experiments of $[^{125}\text{I}]\text{ProTx-II}$ 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 $[^{125}\text{I}]\text{ProTx-II}$. Initial studies were performed in 384-well format, and subsequently the assay was optimized for 1536-well plates.

Fig. 4. Surface plasmon resonance (SPR) binding assay for DII S1–S4 chimera. (A and B) Biacore T200 SPR sensorgrams showing binding profiles of his-tagged ProTx-II (A; 100 nM) or Huwentoxin-IV (B; 1000 nM) to biotinylated Na_vAb (black), DII S3–S4 (blue), or DII S1–S4 (red). (C) Binding of a duplicate 2-fold concentration series (7.8–1000 nM) of ProTx-II to DII S1–S4. Each color indicates a separate concentration in the series. (D) Binding of a duplicate 2-fold concentration series (7.8–1000 nM) of Huwentoxin-IV (62.5–1000 nM) to DII S1–S4. Each color indicates a separate concentration in the series. Blank buffer injections as well as background binding to a Neutravidin-only flow cell have been subtracted from all displayed sensorgrams.

Optimization for protein and [¹²⁵I]ProTx-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 μ l).

The signal-to-background ratio increased slightly over the first hour of incubation, was then 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 ($IC_{50} = 1.3$ μ M) compared with ProTx-II (data not shown).

Discussion

Na_v1.7 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 Na_v1.7 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 Na_v1.7 DII S1–S4 region with the bacterial Na_v channel Na_vAb, 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 Na_vAb, there is a positive shift in activation of

Fig. 5. Scintillation proximity assay (SPA) with DII S1–S4 chimera in 1536-well format. (A) Competition of the SPA luminescence signal (relative light units, RLU) by cold ProTx-II after a 4-hour incubation. Symbols are the mean $\pm$ S.D. of four replicate wells. The solid line is a four-parameter fit with an IC_{50} of 107 nM and Hill slope of 0.95. (B) Plot of the pIC_{50} of competition by cold ProTx-II versus time. (C) Plot of the signal-to-background ratio versus time. (D) Plot of the assay Z' statistic versus time. The signal-to-background ratio and Z' statistic were calculated using two columns each (32 wells) for the assay high and low signals.

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 Na_VAb , 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 $\text{Na}_V1.2$ 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 $\text{Na}_V1.7$ 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 $\text{Na}_V1.2$ is important for ProTx-II inhibition (Sokolov et al., 2008).

In contrast, mutation of residues in DII S1–S2 of $\text{Na}_V1.5$ did not significantly affect ProTx-II inhibition (Smith et al., 2007). We find that transfer of $\text{Na}_V1.7$ DII S3–S4 helix-turn-helix motif to Na_VAb 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 $\text{Na}_V1.7$ DII S1–S2 was also required. These results suggest that in the context of Na_VAb template additional residues of $\text{Na}_V1.7$ 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 ($K_d = 30$ nM), the binding is weaker than that observed with wild-type human $\text{Na}_V1.7$ expressed in HEK293 cells ($K_d = 0.3$ 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 $\text{Na}_V1.7$ (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 Na_V 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 $\text{Na}_V1.7$ 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 $\text{Na}_V1.7$ 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 Na_VAb) 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 to 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 $\text{Na}_V1.7$ in a functional assay.

SPR is a powerful method to study protein-peptide interactions in detail. Alpha-scorpion toxin binding to isolated $\text{Na}_V1.2$ VSD4 can be detected by SPR (Martin-Eaucclair 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 Na_VAb or the DII S3–S4 chimera, confirming that the SPR signal reflects a specific interaction with the $\text{Na}_V1.7$ 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 $\text{Na}_V1.7$ DII chimera with the bacterial channel Na_VAb . The chimera forms functional channels and is sensitive to the $\text{Na}_V1.7$ 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 $\text{Na}_V1.7$. Furthermore, this chimera approach may have broad applicability

for enabling screening at other membrane protein targets of pharmaceutical interest.

Authorship Contributions

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

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

Contributed new reagents or analytic 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, Watson, Ahljianian, Bristow, Herrington.

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Address correspondence to: Dr. Ramkumar Rajamani, Bristol-Myers Squibb, 5 Research Parkway, Wallingford, CT 06492. E-mail: ramkumar.rajamani@bms.com