Published in final edited form as:

ACS Chem Biol. 2018 September 21; 13(9): 2568–2576. doi:10.1021/acscchembio.8b00513.

α 1-FANGs: Protein Ligands Selective for the α -Bungarotoxin Site of the α 1-Nicotinic Acetylcholine Receptor

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Abstract

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels that play a central role in neuronal and neuromuscular signal transduction. Here, we have developed FANG ligands, fibronectin antibody-mimetic nicotinic acetylcholine receptor-generated ligands, using mRNA display. We generated a 1 trillion-member primary e10FnIII library to target a stabilized α 1 nicotinic subunit (α 211). This library yielded 270000 independent potential protein binding ligands. The lead sequence, α 1-FANG1, represented 25% of all library sequences, showed the highest-affinity binding, and competed with α -bungarotoxin (α -Btx). To improve this clone, a new library based on α 1-FANG1 was subjected to heat, protease, binding, off-rate selective pressures, and point mutations. This resulted in α 1-FANG2 and α 1-FANG3. These proteins bind α 211 with K_D values of 3.5 nM and 670 pM, respectively, compete with α -Btx, and show improved

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The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.8b00513.

Plot of $G_{unfolding}$ versus inverse temperature of α 1-FANG1 and its derivatives (Supplemental Figure 1) and determination of α 1-FANG3 K_{on} and K_{off} rates via radiolabeled binding assays (Supplemental Figure 2) (PDF)

subunit specificity. $\alpha 1$ -FANG3 is thermostable ($T_m = 62\text{ }^{\circ}\text{C}$) with a 6 kcal/mol improvement in folding free energy compared with that of the parent $\alpha 1$ -FANG1. $\alpha 1$ -FANG3 competes directly with the α -Btx binding site of intact neuromuscular heteropentamers [$(\alpha 1)_2\beta 1\gamma\delta$] in mammalian culture-derived cellular membranes and in *Xenopus laevis* oocytes expressing these nAChRs. This work demonstrates that mRNA display against a monomeric ecto-domain of a pentamer has the capability to select ligands that bind that subunit in both a monomeric and a pentameric context. Overall, our work provides a route to creating a new family of stable, well-behaved proteins that specifically target this important receptor family.

Graphical Abstract

FANG: Fibronectin Antibody-mimetic NAChR Generated ligand

Nicotinic acetylcholine receptors (nAChRs) make up a superfamily of ligand-gated ion channels located at both pre- and postsynaptic junctions.¹ nAChRs are a subset of the Cys-loop receptor family that also includes GABAA, serotonin, and glycine receptors.^{2,3} nAChRs are composed of homo- or heteropentamers. One distinction among heteropentameric nAChRs is neuronal type and muscle type. The muscle-derived nAChR is composed of two $\alpha 1$, $\beta 1$, γ , and δ or ϵ subunits, while the neuronal heteropentamers contain only α ($\alpha 2$ –10) and β ($\beta 2$ –4) subunits.^{4,5} In all nAChRs, acetylcholine (ACh) binds at the interface between an α subunit and an adjacent non- α subunit. The α subunit forms most of the contacts with ACh and is termed the “primary” contributor to the binding interface. ACh binding activates the gate of the receptor, revealing the open channel on the axis of the pentamer.⁶ At the muscle receptor, several existing drugs target the $\alpha 1$ -containing interface and suppress spasms during anesthesia.^{7–9}

The standard reagent for binding and recognition of the extracellular domain of $\alpha 1$ is α -bungarotoxin. α -Btx is an 8 kDa toxin isolated from the Taiwanese banded krait (*Bungarus multicinctus*), and similar toxins occur in other elapid snakes. These toxins have a three-finger tertiary structure that uses four disulfide bonds to maintain the toxin structure.¹⁰ The tip of one finger inserts into the agonist binding site of $\alpha 1$.¹¹ This toxin stabilizes an inactive conformation of the $\alpha 1$ subunit, blocking responses to the agonist, blocking nerve–muscle transmission, and paralyzing the snake’s prey.^{5,12–14} α -Btx is highly stable and has a high affinity for $\alpha 1$ ($K_D = 0.2\text{ nM}$), rendering it one of the most useful reagents for studying $\alpha 1$.¹⁵ α -Btx was crucial in obtaining the crystal structure of the extracellular domain of

mouse $\alpha 1$ because the toxin stabilized the normally dynamic C loop, which is vital for obtaining resolvable crystals of the $\alpha 1/\alpha$ -Btx complex.¹⁶ The elapid toxins appear to have evolved in the snake salivary gland from members of the lynx/Ly6 family.¹⁷

Despite its widespread use in research, α -Btx is an imperfect tool for the study of $\alpha 1$. α -Btx binds $\alpha 1$ but also binds to paralogous nAChR subunits $\alpha 7$ and $\alpha 9$. The affinity of α -Btx for $\alpha 7$ ($K_D = 1.8$ nM¹⁸) is similar to that for $\alpha 1$ ($K_D = 0.27$ nM¹⁶), while the level of binding of α -Btx to $\alpha 9$ ($K_D = 66$ nM¹⁹) is lower but still significant.²⁰ Additionally, there are no publications about convenient α -Btx synthesis or expression in a renewable system due to the disulfide-rich nature of α -Btx. Other strategies to alter the toxin, such as excision of the loops in α -Btx responsible for contacting $\alpha 1$, produce peptides with specificities and affinities lower than those of the intact toxin.²¹ Also problematic is the fact that the study of nAChR subunits *in vivo* with α -Btx is complicated by its nAChR blockade, which paralyzes test organisms and requires tedious maintenance on a respirator.²² The limitations of α -Btx render it an undesirable starting point for the generation of new $\alpha 1$ binding reagents and argue for the development of new ligands.

Several previous efforts tried to find alternative reagents to α -Btx with improved properties. These approaches included using rabies virus glycoprotein (RVG) and even regions of the gp120 protein of the human immunodeficiency virus (HIV).^{21,23} Unfortunately, these attempts generated only peptides and proteins with specificities similar to and affinities lower than those of α -Btx;^{21,23} consequently, α -Btx has remained the standard reagent for $\alpha 1$ research for several decades.

Here, we used mRNA display to generate novel ligands for $\alpha 1$. We used human fibronectin,²⁴ a scaffold containing variable loops, for our library (Figure 1b). mRNA display enables the design of proteins and peptides via *in vitro* selection and directed evolution experiments using high-complexity libraries (10^{14} independent sequences) in a strictly monomeric format, with precise control over both binding conditions and library sequence content.^{24,25} Our prior work demonstrates that libraries may be constructed using a well-defined protein domain bearing variable loops similar to antibodies.^{24–28} Our goal was to create an $\alpha 1$ reagent (1) with selectivity for the $\alpha 1$ nAChR subunit, (2) with a binding affinity similar to that of α -Btx, (3) that could be genetically encoded for protein expression and *in vivo* experiments,²⁹ and (4) to help determine if *in vitro*-developed ligands would alter the ACh-induced current in the muscle nAChR.

RESULTS AND DISCUSSION

Primary Selection To Generate $\alpha 1$ -FANG1.

The goal of this work was to generate high-affinity, high-specificity, recombinant protein reagents directed at the $\alpha 1$ subunit of the nAChR. To do this, we used an antibody-mimetic library termed e10FnIII in selections against the soluble ecto-domain of $\alpha 1$, which contains three stabilization mutations (V8E/W149R/V155A), termed $\alpha 211$.^{15,16,27,30} Target-specific binding was observed in round 3, with appreciable binding over background in round 6 (Figure 2a). Once appreciable binding over background appeared in radiolabeled pull-down assays, pool 6 was cloned and sequenced; six clones were assayed for function. Clone 6.4,

dubbed $\alpha 1$ -FANG1, exhibited the best binding and comprised approximately 25% of the pool sequences (Figures 1a and 2b). Radiolabeled $\alpha 1$ -FANG1 bound immobilized $\alpha 211$ with the highest percentage among tested sequences, at 14% of total counts introduced. The initial data we obtained motivated us to focus on characterization of $\alpha 1$ -FANG1 and its properties.

Preincubation of α -Btx with $\alpha 211$ immobilized on beads greatly reduced the level of binding of $\alpha 1$ -FANG1 to $\alpha 211$ (Figure 2c), indicating that $\alpha 1$ -FANG1 recognized an epitope similar to α -Btx and had potential as a starting point for generating a new $\alpha 1$ reagent. While $\alpha 1$ -FANG1 expressed in reticulocyte lysate seemed to be highly functional in pull-down experiments, expression in *Escherichia coli* gave rise to aggregation and smeared bands when complexed with $\alpha 211$ in gel shift assays (data not shown). $\alpha 1$ -FANG1 is also sensitive to temperature (Figure 2d).

Maturation Selection Generates $\alpha 1$ -FANG2 and $\alpha 1$ -FANG3.

We sought to improve the affinity, folding stability, and protease resistance of $\alpha 1$ -FANG1. Rather than mutating the scaffold, however, our strategy optimized the protein by varying only the loop sequences. The rationale for this approach was to maintain the conserved human-derived scaffold while more fully exploring the remaining sequence space at our randomized positions.

For the maturation selection, we created a doped library based on the $\alpha 1$ -FANG1 sequence (Figure 2b) where each position was 58% wild type at the DNA level (19–40% wild type at the amino acid level). We then introduced two pressures: thermal denaturation and proteolytic degradation. First, using the data from Figure 2d, we chose a 10 min incubation step at 65 °C as a thermal challenge. Second, we chose Proteinase K treatment, which degrades unfolded protein across a broad range of amino acid residues and digests unfolded protein more rapidly than folded protein,^{31,32} which aided our goal of improved protease resistance and stability.

We performed five rounds of mRNA display with the maturation selection library against $\alpha 211$ immobilized on beads (Figure 3a). At round 3, a 10 min, 65 °C incubation step was added. Round 4 added a 1 min exposure to Proteinase K, and round 5 included an off-rate selection by allowing α -Btx to compete with a fibronectin pool already bound to $\alpha 211$. We observed binding over background in round 2 (Figure 3a). In round 3, heat and protease both inactivated the library; by round 5, however, heat and protease treatment resulted in little loss of library binding. Pools 4 and 5 were cloned and sequenced (Figure 3b); the five most represented clones were assayed for function (Figure 3c). One sequence, $\alpha 1$ -FANG2, exhibited the best resistance to the temperature and proteolytic challenges from selection (Figure 3c). Further analysis of $\alpha 1$ -FANG2 showed a clear binding preference for $\alpha 211$ over the wild-type rat $\alpha 9$ ecto-domain in radioactive pull-down assays (Figure 3d). In this assay, $\alpha 1$ -FANG2 bound $\alpha 211$ at 78% and bound $\alpha 9$ at only 0.5%, a 150-fold difference.

$\alpha 1$ -FANG2 exhibits a stability higher than that of $\alpha 1$ -FANG1 but still showed some tendency toward aggregation. To address this, we constructed several scaffold mutants with the intent of improving the stability. Two of the positions we chose to optimize

were Lys11 and Gln19, which were previously found to improve clone stability.^{24,28} Our characterization identified two point mutations that further stabilized these positions in the e10FnIII scaffold, K11R and Q19E (Figure 1a), giving a final protein less prone to aggregation, α 1-FANG3.

T_m Determination and $G^{\circ}_{\text{unfolding},303\text{ K}}$ Calculations for α 1-FANG Proteins.

With a highly stable protein in hand, we decided to quantify the $G^{\circ}_{\text{unfolding},303\text{ K}}$ for each of our α 1-FANG proteins. We measured the change in molar ellipticity at θ_{222} of purified α 1-FANG1, α 1-FANG2, and α 1-FANG3 protein by circular dichroism (CD) over a range of temperatures to generate melting curves (Figure 4a). We then fit the curves using previously published procedures.³³ The melting temperature for α 1-FANG1 was 45 °C. The T_m for α 1-FANG2 was 69 °C, while α 1-FANG3 exhibited a lower T_m of 62 °C (Table 1). Maturation selection helped select for a protein that was 17–24 °C more thermostable than the parent clone. The dramatic increase in T_m for α 1-FANG2 and α 1-FANG3 indicates that the introduced pressures in selection successfully enhanced stability. Although the T_m of α 1-FANG3 is lower than that of α 1-FANG2, the profile of the T_m for α 1-FANG3 shows a narrower transition zone, characteristic of a larger enthalpy and more cooperative transition.

We also used CD to measure the melting temperature of the enhanced fibronectin scaffold (e10FnIII) that composes our library. For this measurement, we substituted the BC and FG loops of wild-type fibronectin into our enhanced scaffold, which allowed us to have a direct comparison of our clones with something analogous to the native protein. The T_m for e10fnIII is 72 °C. α 1-FANG1 has a T_m lower than that of e10FnIII, but α 1-FANG2 and α 1-FANG3 have melting temperatures within 10 °C of that of the starting scaffold (Table 1).

Using the CD data (Figure 4b), we calculated the $G^{\circ}_{\text{unfolding},303\text{ K}}$ for each α 1-FANG protein (Table 1) using an extrapolation of the CD transition zone to 303 K (Supplementary Figure 1). e10FnIII with wild-type loops had a $G^{\circ}_{\text{unfolding},303\text{ K}}$ of 6.8 ± 1.1 kcal/mol, which is in agreement with the published value of 7.4 ± 0.3 kcal/mol for the

$G^{\circ}_{\text{unfolding},303\text{ K}}$ of wild-type fibronectin 1–7 (wtFnIII 1–7).²⁷ The $G^{\circ}_{\text{unfolding},303\text{ K}}$ for α 1-FANG1 is 3.4 ± 0.9 kcal/mol. The $G^{\circ}_{\text{unfolding},303\text{ K}}$ for α 1-FANG2 is 4.7 ± 0.7 kcal/mol, showing that on its own, maturation selection improved the folding stability of the protein. The $G^{\circ}_{\text{unfolding},303\text{ K}}$ of α 1-FANG2 is 2.1–2.5 kcal/mol lower than that of wtFnIII 1–7 or e10FnIII with wild-type loops. The T_m of 69 °C for α 1-FANG2 echoed these data, as it is slightly lower than the T_m of e10FnIII (72 °C) with wild-type loops. Although α 1-FANG3 had a T_m lower than those of α 1-FANG2 and e10FnIII with wild-type loops, it had a $G^{\circ}_{\text{unfolding},303\text{ K}}$ larger than those of both proteins, with a value of 10.4 ± 1.0 kcal/mol. The introduced point mutation in α 1-FANG3 added stability to α 1-FANG2, indicating a more stable scaffold. The combination of pressures during the selection process and added mutation directly contributed to the high stability of α 1-FANG3.

K_D Determination of α 1-FANG Proteins.

To test the affinity of α 1-FANG2 and α 1-FANG3, we determined the forward (k_1) and reverse (k_{-1}) rate constants for complex formation. α 1-FANG2 bound α 211 with a K_D of 3.5 nM, and α 1-FANG3 bound α 211 with a K_D of 670 pM (Table 1 and Supplementary

Figure 2). The increased stability of the introduced point mutations enhanced the affinity of $\alpha 1$ -FANG3 for $\alpha 211$.

Native Gel Assays To Test $\alpha 1$ -FANG3 Binding of $\alpha 211$ in Free Solution.

To further characterize $\alpha 1$ -FANG3, we tested the ability of $\alpha 1$ -FANG3 to bind $\alpha 211$ in a native gel shift assay. There, $\alpha 1$ -FANG3 incubated with $\alpha 211$ showed a strong band shift when run on a native gel (Figure 5). The gel shift was similar to that given when the $\alpha 211/\alpha$ -Btx complex is run on a native gel, but at a higher level of migration (Figure 5a). After incubation of $\alpha 1$ -FANG3 at 37 °C for 30 min, $\alpha 1$ -FANG3 was still stable and able to bind to $\alpha 211$ as indicated by a strong gel shift, confirming the temperature stability shown in the maturation selection clone binding assays. Simultaneous incubation of a 1:1 α -Btx: $\alpha 1$ -FANG3 molar ratio with $\alpha 211$ resulted in a band shift corresponding to a mixture of the $\alpha 211/\alpha 1$ -FANG3 complex and the $\alpha 211/\alpha$ -Btx complex. $\alpha 1$ -FANG3 thus appeared to bind to $\alpha 211$ with an affinity on par with that of α -Btx. An increase of the α -Btx: $\alpha 1$ -FANG3 ratio resulted in a decrease in the intensity of the $\alpha 211/\alpha 1$ -FANG3 complex band and an increase in the intensity of the $\alpha 211/\alpha$ -Btx complex band. The gel shift assays also confirmed the high specificity of $\alpha 1$ -FANG3 for $\alpha 211$ as $\alpha 9$ did not generate any observable band complex, even at a high $\alpha 1$ -FANG3 concentration (Figure 5b).

Competition of $\alpha 1$ -FANG3 with ^{125}I -Labeled Bungarotoxin in Whole nAChR Pentamer Membranes Derived from a TE671 Cell Line.

We determined whether $\alpha 1$ -FANG3 binds intact muscle-type pentamers in addition to *in vitro*-purified monomers. We assessed the competition between $\alpha 1$ -FANG3 and ^{125}I -labeled α -Btx for binding to intact membrane-bound nAChR muscle-type pentamers purified from a muscle-type nAChR-expressing human medulloblastoma cell line (TE671) (Figure 6). $\alpha 1$ -FANG3 partially inhibited binding of ^{125}I -labeled α -Btx, to ~70% of total binding, with a K_i of 2.7 nM. A relevant characteristic of the TE671 cell line is that nicotine produces an only 50% reduction in the level of ^{125}I -labeled α -Btx binding, even at a 100000-fold molar excess of nicotine (data not shown). Our inability to reach the expected 50% reduction in the level of nAChR radiolabeling may be due to α -Btx binding other surface proteins besides $\alpha 1$. This idea is consistent with our observation that $\alpha 1$ -FANG3 is more specific than α -Btx. On the other hand, α -Btx is known to bind in a 2:1 stoichiometry with the pentamer and $\alpha 1$ -FANG3 may not compete equally at both sites.

Competition of $\alpha 1$ -FANG3 with α -Bungarotoxin in *Xenopus* Oocytes.

It was not obvious whether $\alpha 1$ -FANG ligands would affect the gating of the nAChR pentamer, given that there was no selective pressure during the mRNA display process to generate a ligand with a specific gating effect. To assess the effect of $\alpha 1$ -FANG3 on intact $\alpha 1\beta\delta\gamma$ nAChRs, we studied the well-characterized *Xenopus* oocyte system. We recorded the currents induced by 10 μM ACh following exposure to various ligand incubations (Figure 7). To test the potential effects of $\alpha 1$ -FANG3 on ACh-induced currents, oocytes were preincubated with a 200 nM $\alpha 1$ -FANG3 solution for 3 min and then tested with 10 μM ACh. After a 5 min wash, the same oocytes were incubated with a 50 nM α -Btx solution for 3 min, followed by a second test with 10 μM ACh (Figure 7a). The ACh-induced current decreased by only 6% with $\alpha 1$ -FANG3 incubation, while incubation with α -Btx decreased

the ACh-induced current by 89%, showing that the interaction of $\alpha 1$ -FANG3 with the $\alpha 1\beta\delta\gamma$ nAChR is functionally silent when compared to that of α -Btx.

To assess competition between $\alpha 1$ -FANG3 and α -Btx at $\alpha 1\beta\delta\gamma$ nAChRs in the *Xenopus* oocyte system, two sets of oocytes were recorded for ACh-induced currents. One population was preincubated with a 50 nM α -Btx solution alone for 3 min and then tested with 10 μ M ACh. The second set was preincubated with 200 nM $\alpha 1$ -FANG3 for 5 min and then with a solution of 200 nM $\alpha 1$ -FANG3 and 50 nM α -Btx for 3 min and finally tested with 10 μ M ACh. Both populations were tested at 5 min intervals for ACh-induced current over a 20 min recovery period (Figure 7b). Initial ACh-induced currents for the samples treated with α -Btx alone were 26% of the initial value, while the currents for the population preincubated with $\alpha 1$ -FANG3 prior to co-incubation with $\alpha 1$ -FANG3 and α -Btx were significantly different at 38%. During the washout period, currents increased, as previously noted for the washout of elapid toxins,¹² yet the ACh-induced current for the 200 nM $\alpha 1$ -FANG3/50 nM α -Btx oocytes remained ~12% greater than for α -Btx-only oocytes, showing that $\alpha 1$ -FANG3 had a significant protective effect. Thus, α -Btx blocked 75% of the nAChRs in the absence of $\alpha 1$ -FANG3 but only 62% when the nAChRs were protected by $\alpha 1$ -FANG3. Direct co-application of $\alpha 1$ -FANG3 and α -Btx to oocytes, without $\alpha 1$ -FANG3 preincubation, eliminates this protective effect.

Our mRNA display selection against the soluble ecto-domain of $\alpha 1$, $\alpha 211$, resulted in a ligand with potential as a research tool to further the study of $\alpha 1$. $\alpha 1$ -FANG3 is specific for $\alpha 211$ and is the first ligand able to specifically distinguish between the $\alpha 1$ subunit and other nAChR family members. Additionally, $\alpha 1$ -FANG3 is superior to a natural product reagent such as α -Btx, because it requires no synthesis and can be expressed in *E. coli*, while maintaining an affinity similar to that of α -Btx. These properties are a result of the tunable nature of the e10FnIII scaffold; using affinity maturation and thermal and proteolytic challenges, we improved the $G^\circ_{\text{unfolding},303\text{ K}}$ of our initially selected ligand by ~7 kcal/mol and the T_m by 17 °C. Additionally, $\alpha 1$ -FANG3 targets the α -Btx site of $\alpha 1$ but has no active effect on gating of the nAChR pentamer, making it possible to study the receptor in an unperturbed state. These properties of $\alpha 1$ -FANG3 enhance its potential utility for *in vivo* study of $\alpha 1$ distribution, trafficking, and recycling of $\alpha 1$ at the synaptic junction.

More broadly, our work shows that using a monomeric ecto-domain as a target for *in vitro* selection can yield ligands that bind a monomer individually and in the context of a fully assembled receptor. In general, expression and purification of a genetically encoded monomer are more straightforward than generation of an entire pentamer; therefore, the process laid out in our study provides a simpler path to target other nAChR subunits, as well as to target the larger Cys-loop family for novel ligand generation. mRNA display-generated fibronectins against this family could have better specificity, higher stability, and different functionality compared to those of existing molecules, generating a new category of reagents with which to study this important family of proteins.

METHODS

α 211 Expression and Purification.

The α 211 mouse construct was previously published.¹⁵ Production of the wild-type rat α 9 ECD was identical to that of α 211.

Naïve Selection against α 211.

A naïve DNA library containing 1×10^{12} unique molecules, encoding an enhanced version of the 10th human fibronectin type III domain (e10FnIII) with randomized BC and FG loops, was constructed and cleared of stop codons as previously described.^{27,28,34}

For the first round of naïve selection, 1120 pmol of ligated RNA was translated *in vitro*, which resulted in ~4 copies of the 1×10^{12} library for the affinity enrichment step. Affinity enrichment was performed with 400 pmol of α 211 protein immobilized on M-270 Epoxy Dynabeads (Invitrogen). The selection buffer was phosphate-buffered saline supplemented with 1 mg mL⁻¹ bovine serum albumin (Equitech-Bio), 0.01 mg mL⁻¹ yeast tRNA (Calbiochem), and 0.05% (v/v) Tween 20 (Amresco). In subsequent rounds, 160 pmol of ligated RNA was translated *in vitro* and 100 pmol of immobilized α 211 was used for affinity enrichment.

Binding for naïve selection assays was measured by pull-down assays of RNase (Sigma)-treated, oligo-dT cellulose-purified protein fusions against 100 pmol of immobilized α 211, followed by scintillation counting of [³⁵S]methionine (MP Biochemicals). Radiolabeled binding assays for the naïve selection were performed at room temperature for 1 h.

For α 1-FANG1 competition assays with α -Btx, 300 pmol of α -Btx (Biotium) was preincubated with 100 pmol of α 211 protein immobilized on M-270 Epoxy Dynabeads (Invitrogen) for 1 h prior to incubation with oligo-dT cellulose-purified ³⁵S-labeled α 1-FANG1. The rest of the assay was identical to those previously described.

Identification of clones from naïve selection was achieved with Sanger sequencing after insertion of library DNA into the TOPO-TA vector (Life Technologies).

Affinity Maturation Selection.

For affinity maturation selection, a DNA library was prepared as previously described but with the BC and FG loops doped at 58% representation of α 1-FANG1 at the nucleotide level.^{27,28} The affinity enrichment process was the same as that described for naïve selection. For the first round of the affinity matured selection, 960 pmol of ligated RNA was translated *in vitro*. Affinity enrichment for the first round was performed with 300 pmol of α 211 protein immobilized on M-270 Epoxy Dynabeads (Invitrogen). The selection buffer was identical to that used in naïve selection. In subsequent rounds, 180 pmol of ligated RNA was translated *in vitro* and only 100 pmol of immobilized α 211 was used for affinity enrichment. FLAG affinity purification was added in rounds after the first round as previously described. In the third round, a 65 °C incubation at 10 min was added after FLAG purification. In the fourth round, incubation with 0.05 unit of Proteinase K on agarose (Sigma) for 1 min was added after the 65 °C incubation. In the fifth round, an off-rate

selection step was introduced by addition of 300 pmol of α -Btx (Biotium) in selection buffer for 10 min to the affinity-enriched beads after the incubation and removal of the library.

Binding was measured by a pull-down assay of RNase (Sigma)-treated, oligo-dT cellulose-purified fusions against 100 pmol of immobilized α 211 by scintillation counting of [35 S]methionine (MP Biochemicals). Radiolabeled binding assays for the maturation selection were performed at room temperature for 1 h.

For binding assays involving α 9, rat α 9 was immobilized on M-270 Epoxy Dynabeads (Invitrogen). Assays used 100 pmol of immobilized α 9. α 1-FANG2 RNA was reticulocyte translated (Green Hectares) *in vitro* and purified using anti-FLAG M2 agarose beads (Sigma). FLAG-purified α 1-FANG2 was used for binding assays similar to that used for the oligo-dT cellulose-purified protein fusions.

Identification of clones from doped selection was achieved by Sanger sequencing after insertion of library DNA into the TOPO-TA vector (Life Technologies).

On- and off-rate calculations were performed as previously published, but with individual clones rather than entire pools of molecules.³⁵ [35 S]Methionine-labeled *in vitro* reticulocyte-translated (Green Hectares) α 1-FANG proteins were purified using anti-FLAG M2 agarose beads (Sigma). For off-rate calculations, 1×10^6 radioactive counts were incubated with 100 pmol of α 211 immobilized on M-270 Epoxy Dynabeads (Invitrogen) for 1 h. The supernatant was removed, and the beads were washed four times with selection buffer. A 50-fold molar excess of α -Btx (Biotium) in selection buffer was added to the α 211-immobilized beads. At designated time points after introduction, aliquots were removed, and the beads and supernatant were separated and individually counted to provide the relative ratio of bound α 1-FANG protein. For on-rate calculations, 1×10^6 radioactive counts were incubated with 7 pmol of α 211 immobilized on M-270 Epoxy Dynabeads (Invitrogen). At designated time points after introduction, aliquots were removed, and the beads and supernatant were separated and individually counted to provide the relative ratio of bound α 1-FANG protein.

Expression of α 1-FANG1 and Its Derivatives.

α 1-FANG1 was inserted into the pet24a vector (Novagen) containing an N-terminal maltose binding protein (MBP) tag. Sanger sequencing confirmed DNA insertion and the correct reading frame. The fusion protein was expressed using *E. coli* BL-21 (DE3) cultures induced at an OD of ~0.4 with 0.2 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) for 3 h at 30 °C. The proteins were purified with Ni-NTA beads (Qiagen). The MBP fused proteins were cleaved using HRV3C protease (Thermo Scientific); residual MBP was removed with amylose resin (New England Biolabs), and the protease was removed with GST-Sepharose (GE Healthcare).

DNA inserts containing the sequences of α 1-FANG2 and α 1-FANG3 were subcloned into the pET 24a vector (Novagen). Sanger sequencing confirmed DNA insertion and the correct reading frame. The constructs were transformed into Rosetta 2(DE3)pLysS competent cells with the heat shock method. The transformants were plated on 2XYT agar plates containing

50 $\mu\text{g mL}^{-1}$ kanamycin. Plates were incubated at 37 °C overnight. In each case, four colonies were screened for expression on a small scale, followed by larger-scale protein purification. A pre-inoculate was prepared by seeding a single colony in 50 mL of 2XYT medium with kanamycin. The culture was incubated at 37 °C while being shaken (220 rpm) overnight. One liter of inoculate was set using 10 mL of the overnight pre-inoculate, at 37 °C. When the OD₆₀₀ reached 0.6–0.8 (2.5–3 h), cells were induced at 1 mM IPTG for 3 h at 37 °C. Cells were then harvested by centrifugation at 6000g for 20 min at 4 °C. Cell pellets were stored at –20 °C until they were purified.

Frozen cells were thawed on ice and resuspended with Ni-NTA binding buffer [50 mM NaH₂PO₄ (pH 7.8), 0.5 M KCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, and 20 mM imidazole] containing protease inhibitors (1 mM PMSF, 1 $\mu\text{g mL}^{-1}$ leupeptin, and 1 $\mu\text{g mL}^{-1}$ pepstatin A). Then, cells were lysed with sonication followed by centrifugation at 18000 rpm for 30 min at 4 °C. The supernatant was incubated with Ni-NTA agarose beads (Qiagen) at 4 °C for 2–3 h with end-over-end rotation. The protein was eluted with elution buffer [50 mM NaH₂PO₄ (pH 7.8), 0.5 M KCl, 10% (v/v) glycerol, and 500 mM imidazole] after being washed with binding buffer to remove loosely bound proteins. The eluted protein was concentrated and diluted with Mono Q buffer A [20 mM HEPES (pH 7.5)] that did not contain any salt to lower the salt concentration for anion exchange chromatography. The final salt concentration was adjusted to 100–120 mM. Some precipitations were observed, and they were removed by centrifugation at 10000g for 10 min or by filtration using a 0.22 μm filter. The sample was subjected to an affinity column (Mono Q HR 5/5, GE Healthcare) to purify proteins using a salt gradient from 0 to 100% buffer B (Mono Q buffer B contains 1 M NaCl). Bound peak fractions and flow-through fractions were separately concentrated and run over a size exclusion column (Superdex 75 10/300 GL, GE Healthcare) using 20 mM HEPES (pH 7.5) and 300 mM NaCl buffer. Each peak was identified and confirmed by OD₂₈₀ and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels, and fractions of the peaks were pooled and concentrated for future experiments. The final amount of monomer α 1-FANG3 was 1–2 mg from a 1 L culture.

Gel Shift Assays.

Mouse α 211 was mixed with α 1-FANG3 or α -Btx at an equimolar ratio (1 μg of α 211 was used), and the mixture incubated on ice for 1 h (except for the competitive assay). For the competitive assay, the amount of α -Btx was increased relative to the amount of α 1-FANG3 (α -bungarotoxin: α 1-FANG3 ratios of 1:1, 2:1, 5:1, and 10:1), and the samples were allowed to equilibrate for 16 h overnight. Heated samples were prepared by heating α 1-FANG3 in a heat block for 37 °C for 30 min, and samples were cooled on ice before the addition of mouse α 211. Just before samples were loaded into a gel, 5 $\times$ loading buffer (containing no dye) was added to each sample tube, and samples were loaded on a 10% native-PAGE gel at 4 °C. The gel was run for 3–3.5 h at 100–120 V and ~15 mA in a 4 °C room with TBE buffer. Bands were detected with Coomassie staining.

Circular Dichroism (CD) Spectroscopy.

Ultraviolet–CD spectra data were acquired with a Jasco J815 spectropolarimeter (at the University of Southern California NanoBiophysics Core Facility) equipped with a Peltier

temperature-control device. The buffer consisted of 20 mM sodium phosphate, 1 M NaCl, and 6.3 M urea (pH 7). The *E. coli*-expressed $\alpha 1$ -FANG2, $\alpha 1$ -FANG3, and e10FnIII with wild-type loops were measured at a concentration of 25 μ M. $\alpha 1$ -FANG1 was at a concentration of 12.5 μ M. Measurements used a 1 mm path length at 222 nm. The temperature range was 30–95 °C for $\alpha 1$ -FANG2, $\alpha 1$ -FANG3, and e10FnIII with wild-type loops and 4–95 °C for $\alpha 1$ -FANG1. The temperature was increased at a rate of 1 °C min⁻¹. The buffer-only blank was subtracted. Total ellipticities were converted to mean residue molar ellipticities (degrees square centimeters per decimole) before calculation of $G^{\circ}_{\text{unfolding},303\text{ K}}$ and T_{melt} using previously published procedures.³³

Competition of $\alpha 1$ -FANG3 with ¹²⁵I-Labeled Bungarotoxin in Whole nAChR Pentamer Membranes Derived from a TE671 Cell Line.

Cells of human medulloblastoma clonal line TE671/RD (obtained from G. Crawford, City of Hope Research Institute, Duarte, CA, or from the American Type Culture Collection) have been maintained in our laboratory for years according to established protocols. Cells are cultured in Dulbecco's modified Eagle's medium buffered with sodium bicarbonate, containing 4 mM glutamine and 4.5 g L⁻¹ glucose, and supplemented with 10% (v/v) horse serum and 5% (v/v) fetal calf serum, penicillin (100 units mL⁻¹), and streptomycin (100 μ g mL⁻¹).

Cells were grown to confluence in 100 mm $\times$ 20 mm tissue culture dishes (Sarstedt) and then harvested with a polypropylene spatula into polycarbonate centrifuge tubes. After centrifugation at 4000g, cell culture medium was replaced with 5 mM Tris (pH 7.5) at 0 °C, and cells were homogenized with a Polytron PT2000 for 30 s. The homogenate was centrifuged at 40000g for 10 min, resuspended in Ringer solution with sodium azide (RAZ, 33 mM Tris, 115 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1.3 mM MgSO₄, and 0.1 g/L NaN₃), centrifuged a second time, and then resuspended in RAZ at a concentration of 0.1 mL. These membrane preparations can be stored at 4 °C for months without degradation of nAChR binding sites.

Reaction mixtures (200 μ L) were prepared in RAZ supplemented with 0.1% (w/v) protease-free bovine serum albumin (Jackson ImmunoResearch) with varying concentrations of $\alpha 1$ -FANG3 competing with 10 nM [¹²⁵I]-Tyr⁵⁴- α -bungarotoxin (PerkinElmer). Nonspecific binding was defined with reaction mixtures containing 1 μ M α -cobratoxin. After incubation for 6 h on an orbital shaker at approximately 20 °C, binding reaction mixtures were filtered under partial vacuum through 25 mm GF/C filters (Whatman) pretreated for 30 min in 0.3% (w/v) polyethylenimine (Sigma) and 5% (w/v) powdered skim milk. Borosilicate glass test tubes and GF/C filters were rinsed three times with ice-cold RAZ. Filters were incubated overnight in scintillation cocktail and counted using a Packard TriCarb 2800 TR instrument. Specific counts were normalized to controls, and data were fitted (GraphPad Prism 5.0) to a K_i value assuming single-site binding competition and an α -bungarotoxin K_D of 0.27 nM.¹⁶

Competition of $\alpha 1$ -FANG3 with α -Bungarotoxin in *Xenopus* Oocytes.

Stage VI oocytes from *Xenopus laevis* were isolated and maintained at 16 °C in ND96 solution [96 mM NaCl, 2 mM KCl, 1 mM Mg₂Cl₂, 1.8 mM CaCl₂, and 5 mM HEPES (pH

7.5)] supplemented with 2.5 mM sodium pyruvate (Acros Organics), 50 $\mu\text{g/mL}$ gentamicin (Sigma), and 0.6 mM theophylline (Sigma). Oocytes were injected with a 50 nL sample containing 2 ng of a wild-type nAChR $\alpha/\beta/\delta/\gamma$ mRNA subunit mixture at a 2:1:1:1 ratio.

Voltage-clamp currents from oocytes were recorded, ~24 h after injection, using two-electrode circuits (OpusExpress 6000A, Molecular Devices, Axon Instruments). The pipet microelectrodes were filled with 3 M KCl and had resistances of 0.2–10 M Ω . Oocytes were perfused continuously with Ca^{2+} -free ND96, with additions otherwise noted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We would like to thank Caitlin Nichols for her acronym expertise and Dr. Matthew Mulcahy for his digital prowess.

Funding

Supported by National Institutes of Health Grants R01 AI085583 and R01 DA037161.

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Figure 1.
 α 1-FANG protein sequences and library design. (a) α 1-FANG protein sequences. (b) An mRNA display fibronectin selection library contains a promoter sequence (Δ TMV), a constant scaffold with two randomized regions (BC loop and FG loop), and a covalent linkage via puromycin to the nascent fibronectin protein.

Figure 2.

Naïve fibronectin selection against $\alpha 211$. (a) mRNA display selection for the ecto-domain of $\alpha 211$. Binding over background was apparent at pool 3 and reached an appreciable level by pool 6. (b) Radioactive pull down of selected pool 6 fibronectin clones against 100 pmol of $\alpha 211$. We dubbed the clone with the highest level of binding $\alpha 1$ -FANG1. (c) Competition of $\alpha 1$ -FANG1 with α -Btx. ^{35}S -labeled $\alpha 1$ -FANG1 binds beads with immobilized $\alpha 211$; preincubation of α -Btx with $\alpha 211$ -immobilized beads causes a decrease in the number of detectable counts of radiolabeled $\alpha 1$ -FANG1. (d) $\alpha 1$ -FANG1 performance under temperature stress. Pull down of radiolabeled $\alpha 1$ -FANG1 against $\alpha 211$ shows $\alpha 1$ -FANG1 inactivation at high temperatures.

Figure 3.

Affinity maturation and stabilization of $\alpha 1$ -FANG1. (a) Rounds 1–5 of the maturation selection show increased resistance to introduced pressures as the rounds proceed. Rounds 3–5 were exposed to heat in the form of a 65 °C incubation. Rounds 4 and 5 were exposed to Proteinase K (Pro-K). Round 5 included an off-rate competition with α -Btx. (b) Sequence information for clones derived via maturation selection, with $\alpha 1$ -FANG2 highlighted. (c) Behavior of individual clones from $\alpha 1$ -FANG1-based maturation selection. Individual clones show a range of resistance to the various selective pressures. (d) Comparative pull downs against $\alpha 211$ and the ecto-domain of rat $\alpha 9$ show that $\alpha 1$ -FANG2 is highly selective for $\alpha 211$ over $\alpha 9$.

Figure 4. Melting curves of $\alpha 1$ -FANG ligands. (a) Melting curves for $\alpha 1$ -FANG1, $\alpha 1$ -FANG2, and $\alpha 1$ -FANG3 scaffolds at θ_{222} . $\alpha 1$ -FANG3 shows the most defined transition between two folded states. (b) Folding curves for the clones mentioned above showing the transition from folded to unfolded states.

Figure 5.

Native gel assays show $\alpha 1$ -FANG3 binds $\alpha 211$ in solution. (a) Assay of binding of $\alpha 1$ -FANG3 and α -Btx to $\alpha 211$. The $\alpha 211:\alpha$ -Btx and $\alpha 211:\alpha 1$ -FANG3 ratios are 1:1 except in lanes 6–9 [$\alpha 211:\alpha$ -Btx: $\alpha 1$ -FANG3 ratios of 1:1:1 (lane 6), 1:2:1 (lane 7), 1:5:1 (lane 8), and 1:10:1 (lane 9)]. $\alpha 211$ (lane 1), $\alpha 1$ -FANG3 (lane 2), and their complex (lane 3) show distinct mobilities under native gel conditions. Heating at 37 °C for 30 min does not affect the $\alpha 211/\alpha 1$ -FANG3 complex (compare lanes 3 and 4). The $\alpha 211/\alpha$ -Btx complex (lane 5) can also be distinguished from the $\alpha 211/\alpha 1$ -FANG3 complex on the gel. In direct competition for binding to $\alpha 211$, a 1:1 α -Btx: $\alpha 1$ -FANG3 ratio gives a gel shift that corresponds to both the $\alpha 211/\alpha 1$ -FANG3 complex and the $\alpha 211/\alpha$ -Btx complex (lane 6). Increasing the α -Btx: $\alpha 1$ -FANG3 ratio upon co-incubation with $\alpha 211$ decreases the intensity of the $\alpha 211/\alpha 1$ -FANG3 complex band and increases the intensity of the $\alpha 211/\alpha$ -Btx complex band (compare lanes 7–9). (b) $\alpha 1$ -FANG3 binds $\alpha 211$ specifically with no binding to related family member $\alpha 9$. The $\alpha 1$ -FANG3: $\alpha 9$ and $\alpha 1$ -FANG3: $\alpha 211$ ratios are 1:1, except in lanes 4 and 7 (2:1). $\alpha 9$ (lane 5) shows no complex with $\alpha 1$ -FANG3 (lane 6), while distinct mobilities for $\alpha 211$ (lane 1), $\alpha 1$ -FANG3 (lane 2), and their complex (lane 3) as seen in panel a remain.

Figure 6. $\alpha 1$ -FANG3 competes with ^{125}I -labeled α -Btx for binding to the intact pentamer in membranes. An $\alpha 1$ -FANG3 competition experiment with 10 nM ^{125}I -labeled α -Btx is fitted by a K_i of 2.9 nM and maximally inhibits to ~70% of the total signal.

Figure 7.

$\alpha 1$ -FANG3 protects against α -Btx inhibition of ACh-induced current in the *Xenopus laevis* oocyte expression system. (a) Preincubation of oocytes (n = 8) with 200 nM $\alpha 1$ -FANG3 for 3 min prior to testing with 10 μ M ACh reduces the ACh-induced current by ~5%. Preincubation of the same oocytes with 50 nM α -Btx for 3 min prior to additional testing with 10 μ M ACh reduces the ACh-induced current by ~90%. (b) Preincubation of oocytes with 200 nM $\alpha 1$ -FANG3 for 5 min and then a mixture of 200 nM $\alpha 1$ -FANG3 and 50 nM α -Btx for 3 min shows a protection of ACh-induced current compared to incubations with α -Btx alone. Error bars represent the standard error of the mean (SEM). *** = p value 0.01

Table 1. T_m and $G_{\text{unfolding},303\text{ K}}$ for e10FnIII and $\alpha 1$ -FANG Ligands

fibronectin	T_m (°C)	$G_{\text{unfolding},303\text{ K}}$ (kcal/mol)	K_D for $\alpha 211$
wt10FnIII 1–7	no data	7.4 ± 0.3^{27}	not available
e10FnIII, wild-type loops	72	6.8 ± 1.1	not available
$\alpha 1$ -FANG1	45	3.4 ± 0.9	7.9 nM
$\alpha 1$ -FANG2	69	4.7 ± 0.7	3.5 nM
$\alpha 1$ -FANG3	62	10.4 ± 1.0	670 pM