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NaChBac: The Long Lost Sodium Channel Ancestor

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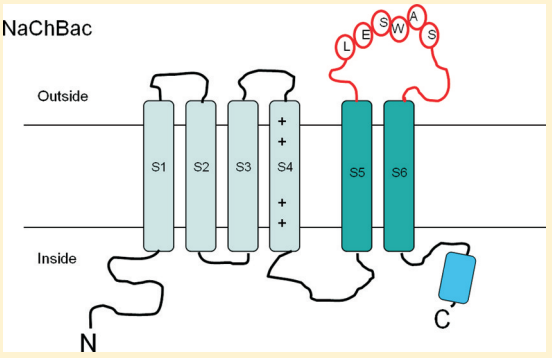
ABSTRACT: In excitable cells, the main mediators of sodium conductance across membranes are voltage-gated sodium channels (Na_Vs). Eukaryotic Na_Vs are essential elements in neuronal signaling and muscular contraction and in humans have been causally related to a variety of neurological and cardiovascular channelopathies. They are complex heavily glycosylated intrinsic membrane proteins present in only trace quantities that have proven to be challenging objects of study. However, in recent years, a number of simpler prokaryotic sodium channels have been identified, with NaChBac from *Bacillus halodurans* being the most well-characterized to date. The availability of a bacterial Na_V that is amenable to heterologous expression and functional characterization in both bacterial and mammalian systems has provided new opportunities for structure−function studies. This review describes features of NaChBac as an exemplar of this class of bacterial channels, compares prokaryotic and eukaryotic Na_Vs with respect to their structural organization, pharmacological profiling, and functional kinetics, and discusses how voltage-gated ion channels may have evolved to deal with the complex functional demands of higher organisms.



Mammalian voltage-gated ion channels function to rapidly conduct ions across membranes. In eukaryotes, sodium channels have evolved to allow sodium flux 15−50 times faster than potassium or calcium ion flux,[1] at a rate of $>10^7$ ions/s, while favoring sodium flux over that of other cations by factors of 100:1 or more.[2,3] There are strong sequence and structural similarities among the sodium-, calcium-, and potassium-specific members of the voltage-gated ion channel family. Although most voltage-gated ion channels include associated regulatory subunits,[4] the α subunit alone is sufficient for functional expression of the channel. Structurally, the α subunits of all voltage-gated ion channels consist of a central pore region, which determines channel selectivity and permeability, and four surrounding voltage sensor regions, which include the binding sites for many of the naturally occurring toxins that bind to sodium channels. Eukaryotic voltage-gated sodium channels (Na_Vs) and voltage-gated calcium channels (Ca_Vs) are large (~300 kDa) heavily glycosylated, monomeric proteins consisting of four homologous domains (designated DI−DIV), with each domain containing six membrane-spanning helices termed S1−S6 (Figure 1). The central pore region, which conducts the ions, is formed by the association of the C-terminal S5 and S6 helices and the intervening "P-loop" from each of the four domains in a pseudosymmetric arrangement. The voltage sensor (VS) subdomains (comprised of the N-terminal S1−S4 transmembrane helices and their connecting loops from each of the domains) are located peripherally with respect to the pore.

The size and complexity of these proteins have proven to be bottlenecks in structural characterization. The most detailed three-dimensional structure for a eukaryotic Na_V is a low-resolution (19 Å) cryo-electron microscopy structure of a channel from the electric eel *Electrophorus electricus*,[5] and the only structures for calcium channels are even lower-resolution (25 and 23 Å, respectively) negatively stained electron microscopy structures of cardiac (L-type)[6] and muscle (T-type)[7] Ca_Vs. The Na_V channel appears to be a bell-shaped molecule ~135 Å long in the transmembrane (TM) direction, with an outer diameter of 100 Å at the cytoplasmic end and 65 Å at the extracellular end.[5] At this resolution, it is not possible to discern any features at even the secondary structure level, much less at the individual atom level, which would be necessary to define functionally important structural details.

In contrast to sodium and calcium channels, both eukaryotic and prokaryotic voltage-gated potassium channels (K_Vs) are simpler structures, being comprised of tetramers of four identical subunits, each of which is roughly one-quarter of the length and corresponds to a single domain of the sodium and calcium channels. Each K_V polypeptide, like each Na_V domain, consists of six transmembrane helices, of which S1−S4 comprise the voltage sensor subdomain and S5−S6 the pore subdomain. The first crystal structure of any ion channel determined[8] was that of the very simple bacterial KcsA channel, which consists of only a pore subdomain. Four of its monomers associate to form a homotetrameric channel. The crystal structure revealed that the organization of the pore is an inverted "tepee"-like structure with inner and outer helices (equivalent to S6 and S5, respectively, of the larger 6TM

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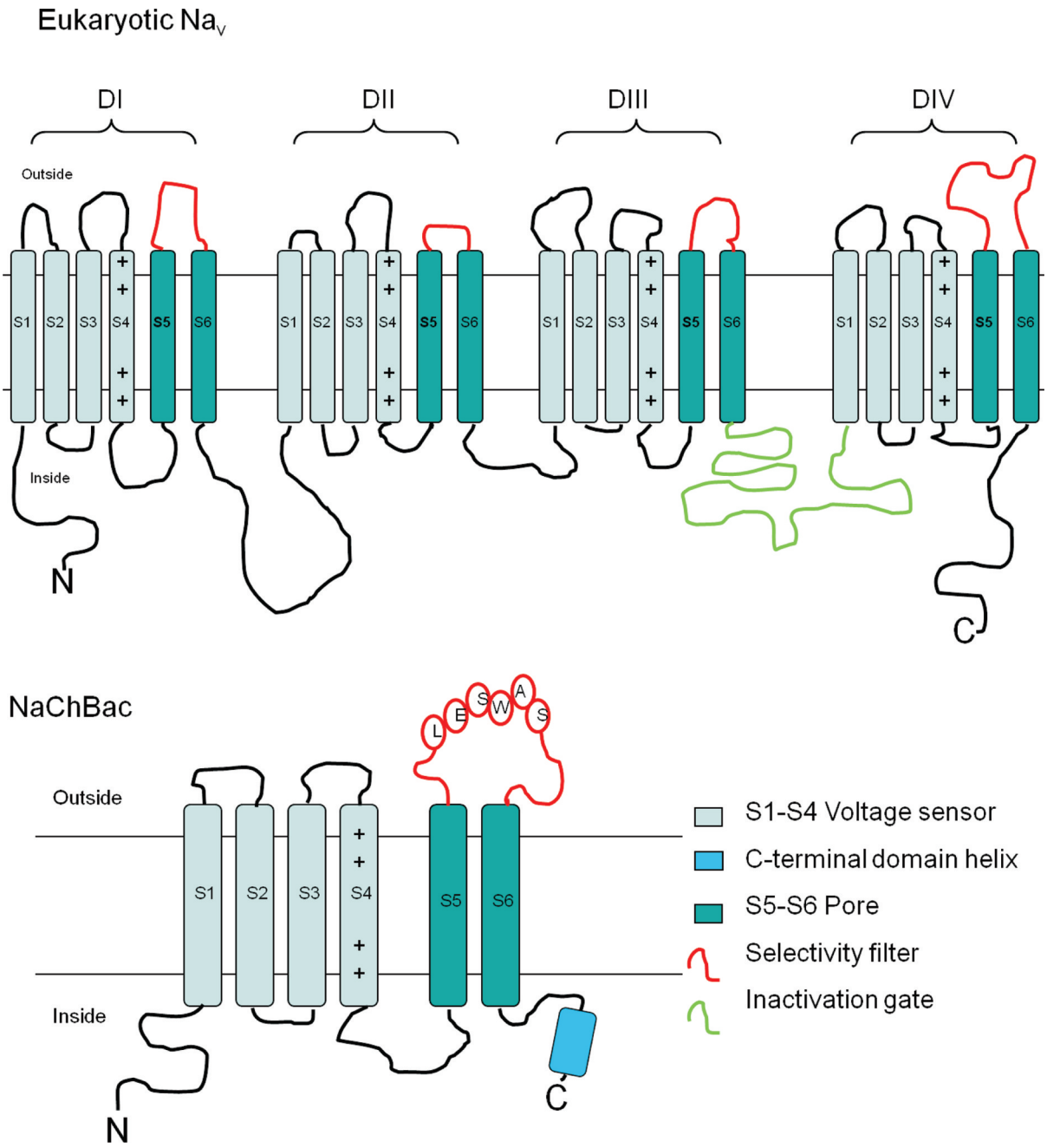

Figure 1. Schematic diagram comparing eukaryotic and prokaryotic voltage-gated sodium channel structures. The four homologous domains in the eukaryotic channel are designated DI–DIV. Transmembrane helices comprising the voltage sensor subdomains (S1–S4) are colored light blue, and the helices (S5 and S6) that form the pore subdomains are colored dark blue. Selectivity filter regions are colored red and interconnecting loops black, and the inactivation gate between DIII and DIV found in eukaryotic channels is colored green. N and C denote the N- and C-termini, respectively.

voltage-gated channels), and a P-loop that provides the ion selectivity filter. The filter is lined by the sequence motif "TVGYG" that is highly conserved among K^+ channels. A somewhat surprising finding was that the ion binding sites involve the backbone carbonyl groups of the amino acids rather than their side chains. Comparisons of the structures of KcsA and another simple bacterial potassium channel, MthK from *Methanobacterium thermautotrophicum*,[9] have suggested they represent closed and open channel conformations, respectively. The MthK channel has splayed bent helices rather than the tepee shape, and it has been postulated[10] that pore gating involves a molecular hinge about a conserved glycine residue. In the case of mammalian K_v channels such as *Shaker*, in addition to this conserved glycine, a proline-containing sequence, PVP, located further along the S6 helix has also been shown to be important for gating.[11] K_VAP from *Aeropyrum pernix* was the first 6TM voltage-gated potassium channel structure determined and contained both VS and pore subdomains.[12] Later the structures of a number of other bacterial and eukaryotic K_Vs[11,13] were determined that also included both subdomains. The conformations of the voltage sensors and their positions relative to the pore differed considerably in the different crystal structures and led to a number of theories regarding the motions involved in the process of voltage sensing; they also

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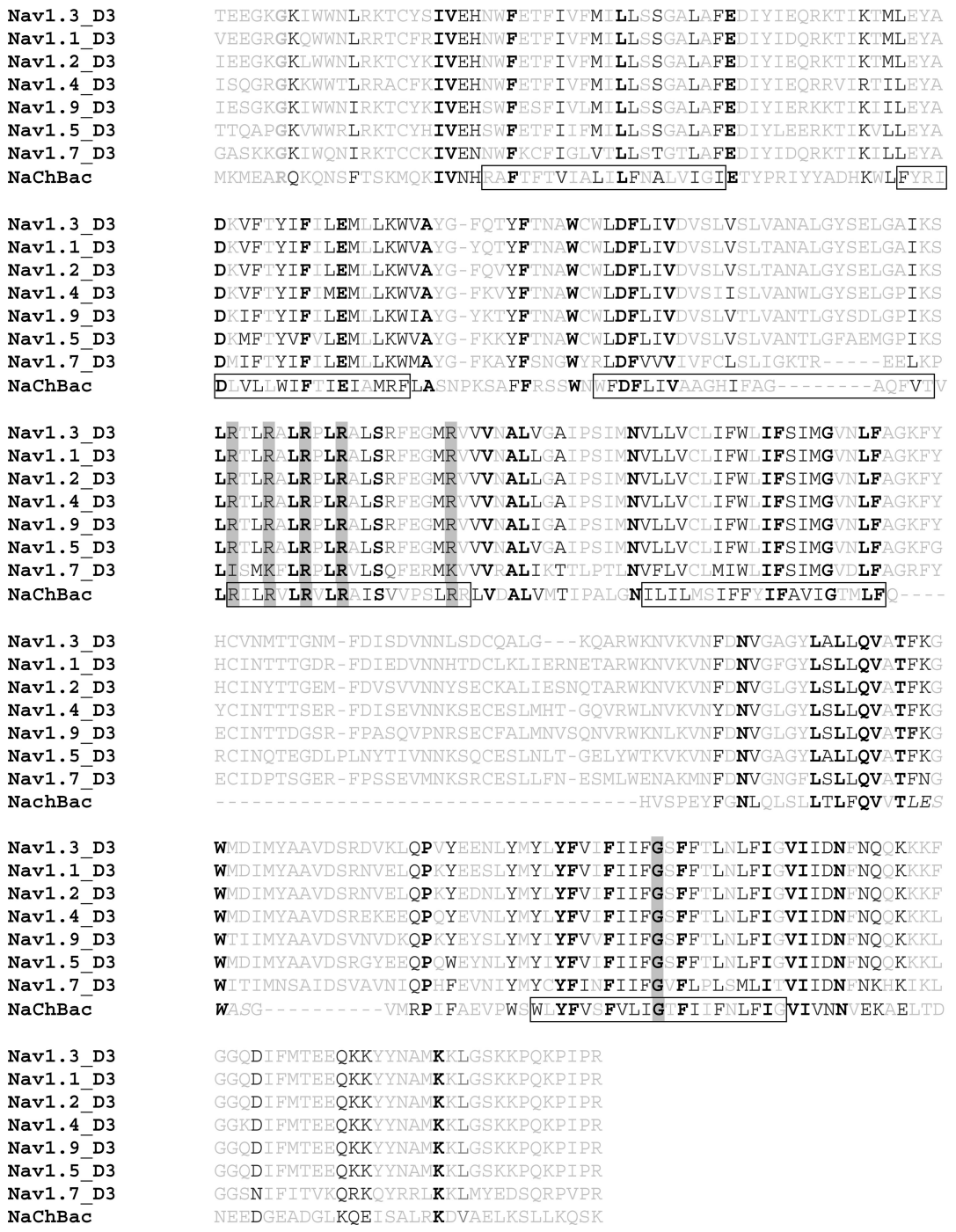

Figure 2. Sequence alignments of the DIII domains of human isoforms $Na_V1.1$ (P35498), $Na_V1.2$ (Q99250), $Na_V1.3$ (Q9NY46), $Na_V1.4$ (P35499), $Na_V1.5$ (Q14524), $Na_V1.7$ (NP002967.2), $Na_V1.8$ (NP_055006.1), and $Na_V1.9$ (Q15858.2) and NaChBac (NP242367). Identical residues are colored black and conserved residues dark gray. The highly conserved arginine residues in the S4 helix are shaded, as is the proposed glycine hinge in S6. The LESWAS filter residues in NaChBac are shown in italics. The transmembrane regions of NaChBac are indicated by the boxes. The alignments were created with CLUSTALW[80] with default parameters, and the locations of the transmembrane regions were estimated using TMHMM.[81]

raised some questions regarding the biological relevance of some of the structures. The determination of additional structures of isolated voltage sensor subdomains and intact channels has improved our understanding of the structure–function relationship of voltage-activated ion channels and added to the ongoing debate.[14−18]

The potassium channel crystal structures have shaped our ideas of what Na_Vs may look like and have led to the production of a number of homology models for them.[19−25] As was the case for potassium channels,[26] the availability of simpler prokaryotic versions of sodium channels is now proving to be important for studies of their structure and function. The first prokaryotic Na_V, NaChBac from *Bacillus halodurans*, was identified in 2001.[27] This was followed by the detection of a number of homologous Na_Vs from other prokaryotic sources.[28−31] Like K_Vs, but unlike eukaryotic Na_Vs, prokaryotic

Na_Vs are smaller single-domain polypeptides that associate to form tetrameric channels. NaChBac is 274 residues long, and in parallel with other voltage-gated ion channels, each chain (which corresponds to a single domain of a eukaryotic Na_V) consists of a voltage-sensing subdomain (formed by helices S1−S4) and a pore subdomain (helices S5 and S6 connected by a P-loop) (Figure 1). The sequence of NaChBac is 17−23% identical to those of various eukaryotic Na_Vs, 18−23% identical to those of eukaryotic Ca_Vs, 12−17% identical to those of K_Vs, and 22−69% identical[28−30] to those of other prokaryotic Na_Vs that have been identified thus far. Although the levels of identity between eukaryotic and prokaryotic Na_Vs are relatively modest, the hydrophobicity profiles and topological organization of all of them are nearly identical, suggesting that the structures of the various channels will be very similar. In addition to the similarities at the primary structure level and conservation of functionally critical residues (Figure 2), it has been shown that the secondary structures of NaChBac and other prokaryotic Na_Vs are very similar to that of one of the eukaryotic Na_Vs (that of the electric eel).[19,32,33] The demonstration that the simple bacterial NaChBac channel could be overexpressed in functional form in heterologous systems has since opened up new opportunities for structure−function studies of voltage-gated sodium channels.

In this review, we describe the features of NaChBac as the exemplar of the prokaryotic sodium channels and compare prokaryotic and eukaryotic Na_Vs with respect to their structural organization and functional characteristics in an attempt to understand how voltage-gated ion channels have evolved to deal with the complex functional requirements of higher organisms.

EXPRESSION OF NACHBAC

In *Escherichia coli*. NaChBac has been successfully expressed in the heterologous bacterium *E. coli*. Strains XL-Gold[34] and DE03 C41[19] support expression, the latter at levels of up to 2 mg of purified protein/L of growth medium.[19,35] Ligand binding and flux assays[19] indicate that following purification in various detergents, NaChBac is correctly folded, sodium selective, and able to bind the channel blocker mibefradil. Thermal melt studies have demonstrated that in the presence of mibefradil, NaChBac is stabilized,[35] similar to the observation that tetrodotoxin is capable of stabilizing eel Na_Vs.[33] The high levels of expression that have been achievable in *E. coli* have been important for the structural studies conducted to date using circular dichroism (CD)[19,31,35] and luminescence resonance energy transfer (LRET) spectroscopies[34] and will be important for future detailed structural X-ray crystallography studies. Furthermore, the ability to reconstitute the detergent-isolated channels into liposomes or other membrane-mimetic systems and the demonstration that they exhibit sodium flux blocked by specific ligands are also important for allowing in vitro functional studies of the isolated protein.[19,36]

In Mammalian Systems. NaChBac has proven to be a good tool for examining Na_V function because of its ability to be expressed in a functional form in Chinese hamster ovary cells,[27] African green monkey kidney cells,[37] and human embryonic kidney cells,[38] which are amenable to single-channel electrophysiology studies. Even though the level of expression in these systems is far too modest for structural studies, they have been very useful for demonstrating structure−function relationships of engineered and mutant proteins in in vivo systems where the protein is not subjected to the rigors of isolation and reconstitution. Although the slow rates of activation, inactivation, and recovery from inactivation and the modest single-channel conductance levels of NaChBac[27] have presented a challenge for the functional characterization of NaChBac channels in reconstituted systems, the ability to express NaChBac in mammalian cells has allowed structure−function characterization of the roles of specific amino acids through a combination of electrophysiology and mutagenesis studies.

Hence, the ability to express functional NaChBac in both prokaryotic and eukaryotics heterologous systems provides a unique opportunity for both structural and functional characterization of this simple sodium channel.

STRUCTURAL ORGANIZATION AND TOPOLOGY OF NACHBAC

Primary Structure. Amino acid sequence analyses reveal that NaChBac and other Na_V bacterial homologues (all of which are single-domain polypeptides) have very high degrees of homology to each other, with levels of sequence identity ranging from 22 to 69%.[28−30] The identity levels with eukaryotic Na_Vs are considerably lower but are still significant. Not surprisingly, NaChBac is more similar to those of many of the lower eukaryotes (23% identical to Na_V from the hydromedusa *Polyorchis penicilatus*) than to those of more highly developed eukaryotes such as electric eel and zebra fish (with both of these, it is 18% identical). For human Na_Vs, NaChBac appears to share a higher level of identity with the DIII domains than with the other domains (Figure 2). In addition to these overall levels of identity, it is noteworthy that many of the functionally critical residues (Figure 2, shaded residues) such as the glycine hinge in S6 and the regularly repeating arginines in S4 are conserved across the prokaryotic−eukaryotic boundary.

Secondary Structure. Circular dichroism spectroscopic studies have compared the secondary structure of NaChBac to that of the eukaryotic Na_V from *El. electricus*.[19,33] Both proteins produce spectra indicative of predominantly α-helical proteins. Analyses of the CD spectra of NaChBac in several different detergents[19,31,35] revealed that it is ∼67% helical, while the eel protein is only ∼50% helical. The higher helical content of NaChBac compared to that of eel Na_V is consistent with the longer connecting loops and termini of eukaryotic Na_Vs, which are predicted to be mostly nonhelical.[33] Other studies have specifically determined the secondary structures of different regions of the protein: For example, more detailed analyses[39] of the NaChBac CD data have shown that the transmembrane helix content is ∼47% (roughly 130 residues, sufficient for 6TM helices), which is consistent with modeling studies that assign 45% of the residues to transmembrane regions of NaChBac.[19] In addition, the secondary structures of its extramembranous C-terminal domain (CTD) residues have been elucidated on a residue-by-residue basis by synchrotron radiation circular dichroism (SRCD) spectroscopy using truncated channel constructs.[31] The 22 distal residues at the C-terminal end of the CTD are α-helical, while the 14 residues closest to the S6 helix do not form regular structures. These findings are in agreement with sequence-based secondary structure predictions[31,40] that suggested that the distal C-terminal helical regions from the four monomers in the tetramer could associate

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to form a coiled-coil helical leucine zipper. Finally, the secondary structure of the pore subdomain, which was determined in a pore-only construct (the C-terminal region starting from the beginning of S5) of one of the NaChBac homologues,[32] showed it to be even more helical than the intact channel; this is because helices S5 and S6 and the C-terminus are highly helical, whereas the full-length channel contains the voltage sensor that has a relatively large nonhelical region in its N-terminus and loops. Hence, by using a combination of CD (or the more sensitive SRCD) spectroscopy and selectively designed constructs, it has been possible to determine in quite fine detail the secondary structures of specific regions of the NaChBac channel, even in the absence of a crystal structure.

Quaternary Structure. On the basis of sequence homology to the individual domains of the eukaryotic Na_Vs, it was proposed that NaChBac would form channels by association among four individual polypeptide chains to produce a symmetric tetramer. Its tetrameric quaternary structure was confirmed by gel filtration, native gel electrophoresis, dynamic light scattering, sedimentation velocity experiments, chemical cross-linking, and electron microscopy studies.[19,31,40] As expected, this is the same oligomeric organization exhibited by the K_Vs. Because all four subunits have identical sequences, NaChBac does not have the potential for separate functional and regulatory roles for different domains, as is possible in eukaryotic Na_Vs where their domain sequences are similar but not identical. (For example, in eukaryotic Na_Vs, just one of the domains, DIV, is specifically associated with certain aspects of inactivation.[41]) In addition, the lack of interdomain loops in NaChBac means that functions such as inactivation by the plug formed by the loop region between domains III and IV in eukaryotic Na_Vs[42,43] must be accomplished in another way in prokaryotic Na_Vs.

Topology. The structural organization of NaChBac has been investigated in further detail using LRET spectroscopy.[34] Measurements of residues at the N-terminal end of the S5 helix (residue 173), in the S5−S6 connecting loop (residue 190), and at the N-terminal end of the S6 helix (residue 208) reveal a common architecture with the pore region of the K_VAP channel and suggest that the three-dimensional structures of the pore subdomains are highly conserved between prokaryotic and eukaryotic members of the entire voltage-gated channel family, not only within the Na_V subfamily. Similar conservation of the voltage sensor subdomain structures across the ion channel family has been implied by spectroscopic[18] and mutagenesis studies.[44,45] In addition, the spatial relationships between the pore and voltage sensor subdomains in NaChBac appear to be similar to those of the *Shaker* channel structure: the S3 and S4 helices of the NaChBac VS subdomain have been shown to be located adjacent to the pore subdomain, while the S1 and S2 helices are at the periphery of the channel. Furthermore, the S4 voltage-sensing helix of one NaChBac monomer is apparently closer to the S5 helix of the neighboring monomer than it is to the S5 helix of the same monomer, based on disulfide cross-linking studies.[45]

Hence, a variety of biophysical studies of NaChBac and homology modeling studies based on potassium channel structures have provided much information at various levels about its structural organization. Very recently, the crystal structure of a NaChBac homologue, NavAb from *Arcobacter butzleri*, in the closed state has been determined.[46] The sequence of this channel is ∼37% identical overall to that of NaChBac, and they are 40% identical in their pore regions. Its structure is consistent with the structural features described above for NaChBac, with the exception that the C-terminus is not visible in the crystal structure, so the spectroscopic and modeling studies remain the only detailed information about that part of the molecule.

STRUCTURE−FUNCTION RELATIONSHIPS OF SPECIFIC REGIONS OF NACHBAC

Voltage Sensor Subdomain. Activation of voltage-gated ion channels occurs through charge movements within the voltage sensor subdomain (helices S1−S4). Similar structures, albeit with very modest levels of sequence identity, have also been found associated with voltage-regulated phosphatases (14% identical to NaChBac)[47] and voltage-activated proton channels (12% identical to NaChBac).[48] These findings along with studies that report the voltage-dependent gating of a non-voltage sensitive channel following the transfer of the VS subdomain from the *Shaker* channel[49] and the observation that isolated VS subdomains can be stably expressed and purified in detergent[16,50] suggest that VSs may have evolved as independent units and are most likely phylogenetically conserved from archaebacteria to mammals. It is these voltage sensors that couple small fluctuations in membrane potential to conformational changes in voltage-gated ion channels, resulting in activation and specific ion flux. It has been postulated that this is accomplished by a positively charged gating region moving with respect to the membrane in response to the electric field. Positively charged amino acids located on the S4 helix (Figure 1), which comprise the most conserved region throughout the entire voltage-gated ion channel family, form four to seven repeated motifs of (predominately) arginine residues separated by two hydrophobic residues. This pattern places the charged residues on a spiralling single surface of the S4 helix. Mutation of these positively charged residues in the S4 helix of the *El. electricus* Na_V showed direct correlation with channel activation.[42] Studies using K_Vs and NaChBac have calculated that three or four charges per voltage-sensing subdomain move during the channel gating cycle.[37,51,52] Sequence analyses reveal that the first four arginine residues in the S4 helix of NaChBac are in positions equivalent to those in the mammalian Na_V voltage sensor subdomains (Figure 2). Mutations that change these positively charged amino acids to neutral ones result in alterations to the kinetics of voltage-dependent activation, observed as a shift in steady state activation toward more depolarized voltages.[38] Mutational[44] and modeling studies[20] of NaChBac support the sliding helix model proposed for the other voltage-gated ion channels, including the involvement of negatively charged amino acid residues located in the S2 and S3 segments in the formation of ion pairs with the S4 helical residues, whereby they stabilize and mediate transmembrane movement of the voltage sensor during activation.[21] Cysteine substitutions in the S4 and S5 helices produced intersubunit disulfide bonds, indicating that T110 and R113 in S4 are in the proximity of residue M164 in S5.[44] Under nonreducing conditions, disulfide-linked mutant channels were unable to generate currents, suggesting that they were locked in a nonconducting state; however, upon reduction of the disulfides, the channels were able to change conformation and became active. Alternatively, changes in the secondary structure (an α-helix to a 3_{10}-helix transition) of the

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S4 helix have been suggested to produce transmembrane movement of the S4 helix, resulting in the more energetically favorable positional changes of the gating charges[14] in potassium channels. Site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy[18] of NaChBac are consistent with residues at the N-terminal end of the S4 helix (residues 110−119) being α-helical and residues 120−128 at the C-terminal end of the S4 helix adopting a 3_{10} hydrogen bonding pattern, and a modeling study[20] has suggested that the N- and C-terminal ends of the S4 helix switching between α-helical and 3_{10}-helical structures could at least partially account for the gating transition.

Potassium channel crystal structures have shown a wide diversity in the juxtapositions between the pore and voltage sensor subdomains.[11−14] That observation suggested that the VS could be an integral unit, which led to the construction of a stand-alone VS subdomain of NaChBac.[18] The voltage sensor construct was capable of folding in the absence of the pore subdomain, and EPR spectroscopic measurements confirmed that its overall fold is more tightly packed than but similar to that of potassium channel voltage sensors.[18] These studies indicate that the VS subdomain folds independently and is stable in the absence of the pore subdomain.

Pore Subdomain. Corresponding studies of a pore-only construct (consisting of the S5 and S6 helices linked by the interconnecting P-loop and the CTD) of the NaChBac homologue Na_VSP (39% identical) have indicated that the pore subdomains can also assemble, fold, and function in a manner independent of the voltage sensor.[32] Unlike the stand-alone voltage sensor constructs, which are monomeric, the pore-only construct forms folded, stable tetramers, but only in the presence of sodium ions. The pore-only construct exhibits sodium permeability and ligand blocking characteristics comparable to those of the full-length channel, thus confirming that the ion translocation function resides in the pore subdomain.

Selectivity Filter. NaChBac ion selectivity is determined by specific residues in the P-loop of its pore subdomain, but unlike prokaryotic K_Vs where the main chain carbonyls determine selectivity,[8] it is the amino acid side chains that are postulated to encode ion selectivity in Na_Vs and Ca_Vs.[53−55] In eukaryotic Na_Vs, four essential residues (DEKA), one from each domain located at equivalent positions in the P-loops, line the pore and impart sodium selectivity. Hence, sodium selectivity was once thought to be a consequence of asymmetry within the pore. Mutation of these residues to an EEEE sequence, the equivalent residues found in Ca_Vs, confers calcium selectivity.[53] It was thus surprising that NaChBac, which is a homotetramer and hence has four identical residues (also glutamates) and is highly homologous in sequence to the signature motif (FxxxTxExW) for Ca_V selectivity regions, exhibits sodium selectivity. Sodium selectivity in NaChBac could be shifted to calcium selectivity by changing the residues in the sequences on either side of the EEEE filter in each of the chains, located at positions 190−195 (LESWAS) (Figure 2).[56] Each mutation would still result in a symmetric selectivity filter. A serine-to-glycine substitution at position 192 (LESWAS to LEGWAS) had no effect on NaChBac permeability, unlike the LESWAS to LESWAD substitution that transformed NaChBac into a nonselective cation channel. Increasing the amount of negative charge resulted in further increased Ca^{2+} selectivity. Mutants LEDWAS and LEDWAD showed greatly increased calcium ion selectivity, which was further increased by the additional substitution of E191D to form LDDWAD. These studies demonstrated that sodium selectivity could be achieved from a homotetramer and thus does not require a four-domain asymmetric pore, as had previously been suggested for eukaryotic Na_Vs. Additionally, it demonstrated that the mere presence of negatively charged residues such as aspartic and glutamic acids in the pore domain does not ensure calcium selectivity.

C-Terminal Domain. The CTDs of eukaryotic Na_Vs are large (~105 residues) cytoplasmic domains involved in channel inactivation through interactions with the loop linking DIII and DIV.[57] Mutations in the CTD alter inactivation kinetics and are directly linked with channelopathies, including cardiac arrhythmia syndromes[58,59] and epilepsy.[60] In eukaryotes, this domain features a calcium ion-binding paired EF hand structure and a distal calmodulin-binding motif.[61,62] In contrast, the CTDs of NaChBac and all other prokaryotic Na_Vs are much shorter (~40 residues). Because NaChBac is a homotetramer and more closely resembles the oligomeric assembly of potassium channels than the monomeric organization of eukaryotic Na_Vs, it was postulated that the CTD of NaChBac might be involved in channel assembly and/or tetramerization,[31] as it is in potassium channels.[63,64] Synchrotron radiation circular dichroism spectroscopy studies demonstrated that the last 22 residues of the CTD are helical while the 14 residues preceding this region are disordered.[31] Hydropathy plots and sequence analyses of the CTD revealed a repeat pattern of hydrophobic and hydrophilic residues typically found in coiled-coil structures.[31,40] Expression studies comparing full-length and CTD-truncated constructs[31] suggested that while the CTD has little effect on overall expression, it influences assembly into the membrane. It is not required for tetramer formation,[40] although it does contribute to stabilization of the tetramers.[31] Hence, the CTDs of prokaryotic and eukaryotic Na_Vs have very different structures, which are reflected in the different functional roles resulting from their different oligomeric states.

FUNCTIONAL PROPERTIES OF NACHBAC

Activation and Inactivation Kinetics. Like other voltage-gated ion channels, NaChBac is activated in response to membrane depolarization and is proposed to undergo several conformational transitions prior to channel opening and subsequent inactivation.[37] However, NaChBac inactivation kinetics do not resemble those of its eukaryotic homologues. Activation and inactivation time constants of 12.9 and 166 ms at −10 mV, respectively, were recorded,[27] which are considerably slower compared with the range of activation and fast inactivation kinetics of eukaryotic Na_Vs (<2 and <10 ms, respectively). Fast inactivation in eukaryotic Na_Vs allows those channels to close and rapidly return to a resting state (the onset of fast inactivation can occur within 1 ms, while return to a resting state takes ~10 ms for fast inactivation, in contrast to >1 s for slow inactivation); the rapid return is essential for the high-frequency propagation of action potentials required in higher-order organisms. Fast inactivation is attributed to the loop region connecting DIII and DIV undergoing a conformational change.[42] NaChBac, as a homotetramer, does not have an equivalent interdomain loop region. K_Vs that are also homotetramers undergo fast inactivation or N-type inactivation through their N-terminal domain that acts like a ball and chain, physically blocking the pore from the intracellular side.[65] This

type of inactivation is unlikely to occur in NaChBac as the amino terminus of NaChBac is too short to produce a "ball and chain" structure. As a result, NaChBac has a rate of inactivation 10−100-fold lower than the range of inactivation rates of eukaryotic Na_Vs, and hence, its inactivation is described as slow or C-type inactivation. The molecular mechanism of slow inactivation is less well understood than that of fast inactivation, but it has been ascribed to conformational changes resulting in collapse of the selectivity filter.[66] Charge-altering substitutions at position 195 in its P-loop altered the rate of inactivation and voltage dependence of NaChBac, whereas mutations in the N-terminal region showed no effect on inactivation rates. This supports the notion that NaChBac undergoes slow inactivation involving the pore region and not the N- or C-terminal domains.[66]

Mutagenesis studies on NaChBac have also examined the role of the proposed glycine hinge at position 219 in S6 of the pore[22,35,67] in gating. In the KcsA crystal structure, the equivalent residue is proposed to provide flexibility to the pore, thus allowing channel opening during activation.[68] In NaChBac, altering the conformationally flexible glycine to a serine resulted in a structure more thermally stable (and presumably rigid) than the wild-type channel;[35] when it was changed to an alanine,[30] inactivation (and, to a lesser extent, activation) was significantly altered. It is notable, however, that the homologue Na_VSheP, which does not possess any glycine residues in its S6 helix, is capable of gating, albeit with a negatively shifted voltage dependence and significantly faster inactivation.[30] Together, these results suggest the glycine is integrally involved in, but not obligatory for, the gating process. Additionally, when this glycine was changed to a conformationally restricted proline residue, which introduces a permanent kink in the S6 helix (perhaps mimicking the open state) and results in significant shifting of the voltage dependence of its activation to more negative voltages, inactivation rates were slowed by 3 orders of magnitude.[22] Thus, the absence of the glycine hinge alters gating but does not prevent it, and the presence of a permanent kink in this position may result in stabilization of the open state in NaChBac.

Pharmacology. Pharmacological profiling has proven to be a powerful tool for structure−function studies of voltage-gated ion channels. The availability of high-affinity, highly specific neurotoxins has allowed the mapping and localization of specific domains and the probing of gating properties of eukaryotic Na_Vs. The wide range of neurotoxins that bind to eukaryotic Na_Vs has been reviewed;[69,70] however, little is known about the binding of toxins to NaChBac, and no NaChBac-specific toxins have yet been identified. Indeed, although it is sodium selective, wild-type NaChBac is insensitive to tetrodotoxin, a toxin known to bind to the extracellular entrance to the pore of eukaryotic Na_Vs.[71] However, NaChBac is inhibited by clinically relevant concentrations of the volatile general anesthetic isoflurane in a concentration-dependent manner like eukaryotic Na_Vs.[72] Hence, NaChBac shares some, but not all, pharmacological properties with eukaryotic Na_Vs.

On the other hand, NaChBac also exhibits some pharmacological properties similar to those of calcium channels. Indeed, the sequence of the NaChBac selectivity filter more closely resembles the signature filter motif (FxxxTxExW) found in Ca_V pore regions,[73] including the conserved glutamates (EEEE), one from each domain, that form the interdomain ring of charged residues, rather than the DEKA ring residues of Na_V pores. Furthermore, NaChBac is specifically blocked by compounds selective for L-type Ca_V channels: NaChBac is most sensitive to the dihydropyridines such as nifedipine and nimodipine with half-inhibitory concentrations (IC_{50}) of 2.2 and 1 μM, respectively.[27] It is affected, but less so, by T-type Ca_V antagonists such as mibefradil (IC_{50} = 22 μM).[27] The sensitivity of NaChBac to dihydropyridines is not obvious from direct comparisons of its sequence with the dihydropyridine binding sites in Ca_Vs. Dihydropyridines are proposed to induce allosteric inhibition of channel opening by binding to the closed conformation of Ca_Vs. Specifically, they are proposed to bind to the S5 helix of DIII, the S6 regions of DIII and DIV, and the P-loop of DIII.[74] On the basis of modeling studies,[20] it was proposed that these hydrophobic blockers bind to the NaChBac residues located in narrow openings from the lipid phase between helices S5 and S6 of one NaChBac monomer and the S6 helix of the adjacent monomer. Specifically, NaChBac residues 151 and 155 of S5, 190 and 191 of the P-loop, and 226 of S6 of one monomer and residues 213, 216, and 224 of the S6 helix of the adjacent monomer form part of the lining of the proposed hydrophobic niche that forms the dihydropyridine binding site. These residues are analogous to residues where mutations in DIII of L-type Ca_Vs inhibit dihydropyridine binding and as such highlight the possibility of a similar hydrophobic niche in both Ca_Vs and prokaryotic Na_Vs.[20] In addition, like L-type calcium channels, NaChBac is relatively insensitive to the N-type Ca_V-binding toxins such as ω-conotoxin GVIA and the P/Q blocker toxin ω-agatoxin.[27] As those toxins have been proposed to bind to the DIII region of the pore and the S3−S4 loop of DIV, respectively, their lack of binding can be used to help define where NaChBac and Ca_V structures are similar and divergent.

It is thus clear that for NaChBac, ion selectivity does not correlate with the pharmacologic sensitivities of other members of the voltage-gated ion channels.

Physiological Roles. Na_Vs are found ubiquitously in eukaryotes.[75] Differences in Na_V activation−inactivation kinetics vary with function and localization. For example, the presence of fast-type sodium channels in the giant axon of cephalopods reflects the highly centralized nervous system necessary for the rapid coordination and execution of motor outputs for high-speed behaviors such as jet-propelled locomotion and high-frequency chromophore operation.[76] In contrast, action potentials in gastropods are ∼10-fold slower than in cephalopods and are associated with slow-type sodium channels that are thought to control pace-making properties.[76] In humans, different isoforms are found in many different tissue types, ranging from skeletal muscle and cardiac myocytes to central and peripheral neurons. They are targets for the treatment of a number of diseases such as long QT-syndrome[58,59] and certain forms of epilepsy[60] and chronic pain.[77] While Na_Vs are not present in all prokaryotes (*E. coli* being a notable example), they are found across the spectrum of both Gram-positive and Gram-negative bacteria, including thermophiles, halophiles, and aerobic, anaerobic, and photosynthetic bacteria.[28−31] However, because they are not ubiquitous, this indicates that they do not perform essential roles for viability in all prokaryotes but rather are involved in specialized functional roles in certain prokaryotes. For example, it has been postulated that prokaryotic Na_Vs may be involved in

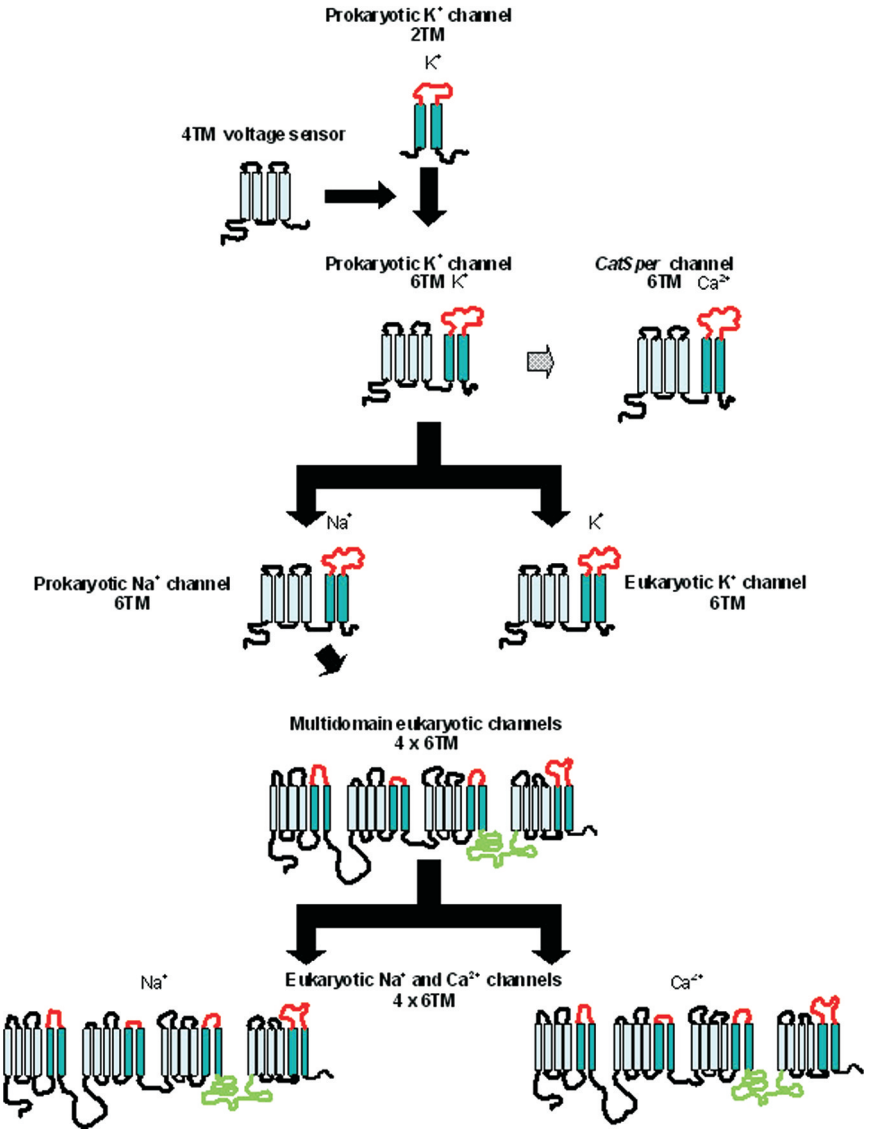

Figure 3. Scheme of the proposed evolutionary relationships among members of the voltage-gated ion channel family.

motility and chemotaxis in marine and alkaphilic species,[27,28] although no such function has yet been demonstrated for NaChBac. However, because *B. halodurans* lives in very high salt conditions, even though the conductance and kinetic parameters of NaChBac are relatively modest, the influx of sodium through the channel in this environment may be very large. As sodium is a driving force for this bacterium's flagellar motor, NaChBac has been proposed to have a role in the control of flagellar activity,[27] obviously not a function required by all eukaryotic cells.

Hence, despite their structural and functional similarities, it is clear that Na_Vs from prokaryotic and eukaryotic sources have different roles in vivo. They display sufficient structural diversity to allow them to play a range of specialized roles in different organisms and throughout different biological niches.

EVOLUTIONARY RELATIONSHIPS OF NACHBAC AND OTHER VOLTAGE-GATED ION CHANNELS

On the basis of levels of sequence identity among and within the sodium, calcium, and potassium members of the voltage-gated ion channel family, and their electrophysiological properties and overall functional roles, there was speculation that Na_Vs arose after K_Vs and Ca_Vs.[75] Because the four domains of the Na_Vs[75] are more identical in sequence to the corresponding domains of Ca_Vs than to each other and have in turn the lowest levels of identity with K_Vs, it was suggested that a single gene gave rise to (first) potassium and (then) calcium channels. The latter resulted from two gene duplications of the common single-domain ancestral gene, and then Na_Vs arose directly from the multidomain Ca_Vs. This evolutionary pattern was supported by their functional roles because the K_Vs and Ca_Vs would be needed for more basic signaling mechanisms, but Na_Vs would be required only in organisms with developed nervous systems allowing fast propagation of action potential in axons.[75] However, these evolutionary relationships were derived prior to the availability of crystal structures of any members of the voltage-gated ion channel family, and before any prokaryotic sodium channels had been identified, information that now suggests some revision to these schema may be appropriate. The presence of single-domain sodium channels from primitive prokaryotic organisms that are more identical in sequence to eukaryotic Na_Vs than to other members of the voltage-gated ion channel family indicates

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they may have diverged from K_Vs at an earlier stage, before gene duplication (in eukaryotes) had taken place. Because they exhibit sequence characteristics common to both Na_Vs and Ca_Vs, this may suggest that they are ancestral to both and may be the missing link between single-domain K_Vs and multidomain Ca_Vs or Na_Vs. It is at this stage that gene duplication may have arisen.

It seems likely that the simple two-transmembrane types of prokaryotic potassium channels such as KcsA led to 6TM prokaryotic K_Vs[78] by combination with a separately evolved four-transmembrane voltage sensor. The prokayotic 6TM channels then led to 6TM single-domain eukaryotic K_Vs. After the emergence of the 6TM K_Vs, this may have led to a single-domain 6TM ancestral prokaryotic Na_V [as of yet, no single-domain prokaryotic Ca_Vs have been identified, although more distantly related single-domain eukaryotic calcium channels (CatSper and TPCs)[79] have been found]. It is suggested that the single-domain 6TM Na_V channels underwent gene duplication events twice, leading to multidomain proteins that then separately evolved into the multidomain 24-transmembrane Na_Vs and Ca_Vs (Figure 3). Of course, this proposed pattern may change in the future with the identification of new prokaryotic and eukaryotic Ca_Vs and Na_Vs with different levels of sequence similarity. Nevertheless, some conclusions can be made on the basis of the presently known channels: it seems likely that the linked four-domain structures of Na_V and Ca_V channels conferred an evolutionary advantage, in the kinetics of channel activation, inactivation, or recovery from inactivation, but it seems less likely that the four-domain structure evolved to provide ion selectivity.

SUMMARY

NaChBac is a well-characterized exemplar of the class of prokaryotic sodium channels, which are simplified versions of the ubiquitous eukaryotic Na_Vs. Its small size, with relatively short loops and termini, along with its ability to be expressed in both mammalian systems (for functional studies) and heterologous bacterial systems (for structural studies) has allowed mutagenesis and reconstitution studies for exploring functional features and makes it an excellent candidate for structural studies.

It is interesting to note that while NaChBac and other bacterial sodium channels exhibit some similarities to eukaryotic Na_Vs, they also have other structural features more in common with other members of the voltage-gated ion channel family rather than with the eukaryotic members of the sodium channel subfamily. Specifically, NaChBac has an oligomeric organization that resembles that of prokaryotic and eukaryotic K_Vs, a pore architecture with a selectivity filter that is highly homologous in sequence to Ca_Vs, and is specifically inhibited by L-type Ca_V antagonists. Hence, this primitive channel also provides the opportunity to further investigate and compare the different molecular bases of ion selectivity and ligand binding between and within the members of the voltage-gated ion channel family.

In conclusion, NaChBac and the other prokaryotic Na_V homologues have helped reshape our thinking about sodium channel structure, function, and evolution.

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ABBREVIATIONS

Ca_V, voltage-gated calcium channel; CD, circular dichroism; CTD, C-terminal domain; DI–DIV, domains I–IV, respectively; K_V, voltage-gated potassium channel; LRET, luminescence resonance energy transfer; Na_V, voltage-gated sodium channel; NaChBac, voltage-gated sodium channel from *B. halodurans*; S1–S6, transmembrane segments 1–6, respectively; TM, transmembrane; 6TM, six-transmembrane; VS, voltage sensor.

REFERENCES

(1) Hille, B. (2001) *Ionic Channels of Excitable Membranes*, Sinauer Associates Inc., Sunderland, MA.

(2) Marban, E., Yamagishi, T., and Tomaselli, G. F. (1998) Structure and function of voltage-gated sodium channels. *J. Physiol. 508*, 647–657.

(3) Catterall, W. A. (2000) From ionic currents to molecular mechanisms: The structure and function of voltage-gated sodium channels. *Neuron 26*, 13–25.

(4) Hanlon, M. R., and Wallace, B. A. (2002) Structure and function of voltage-dependent ion channel regulatory β subunits. *Biochemistry 41*, 2886–2894.

(5) Sato, C., Ueno, Y., Asai, K., Takahashi, K., Sato, M., Engel, A., and Fujiyoshi, Y. (2001) The voltage-sensitive sodium channel is a bell-shaped molecule with several cavities. *Nature 409*, 1047–1051.

(6) Wang, M. C., Collins, R. F., Ford, R. C., Berrow, N. S., Dolphin, A. C., and Kitmitto, A. (2004) The three-dimensional structure of the cardiac L-type voltage-gated calcium channel: Comparison with the skeletal muscle form reveals a common architectural motif. *J. Biol. Chem. 279*, 7159–7168.

(7) Walsh, C. P., Davies, A., Butcher, A. J., Dolphin, A. C., and Kitmitto, A. (2009) Three-dimensional structure of $Ca_V3.1$. Comparison with the cardiac L-type voltage-gated calcium channel monomer architecture. *J. Biol. Chem. 284*, 22310–22321.

(8) Doyle, D. A., Cabral, J. M., Pfuetzner, R. A., Kuo, A. L., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) The structure of the potassium channel: Molecular basis of K^+ conduction and selectivity. *Science 280*, 69–77.

(9) Jiang, Y. X., Lee, A., Chen, J. Y., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) Crystal structure and mechanism of a calcium-gated potassium channel. *Nature 417*, 515–522.

(10) Jiang, Y. X., Lee, A., Chen, J. Y., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) The open pore conformation of potassium channels. *Nature 417*, 523–526.

(11) Long, S. B., Campbell, E. B., and Mackinnon, R. (2005) Crystal structure of a mammalian voltage-dependent Shaker family K^+ channel. *Science 309*, 897−903.

(12) Jiang, Y., Lee, A., Chen, J., Ruta, V., Cadene, M., Chait, B. T., and MacKinnon, R. (2003) X-ray structure of a voltage-dependent K^+ channel. *Nature 423*, 33−41.

(13) Lee, S. Y., Lee, A., Chen, J., and MacKinnon, R. (2005) Structure of the KvAP voltage-dependent K^+ channel and its dependence on the lipid membrane. *Proc. Natl. Acad. Sci. U.S.A. 102*, 15441−15446.

(14) Long, S. B., Tao, X., Campbell, E. B., and MacKinnon, R. (2007) Atomic structure of a voltage-dependent K^+ channel in a lipid membrane-like environment. *Nature 450*, 376−382.

(15) Clayton, G. M., Altieri, S., Heginbotham, L., Unger, V. M., and Morais-Cabral, J. H. (2008) Structure of the transmembrane regions of a bacterial cyclic nucleotide-regulated channel. *Proc. Natl. Acad. Sci. U.S.A. 105*, 1511−1515.

(16) Cuello, L. G., Cortes, D. M., and Perozo, E. (2004) Molecular architecture of the KvAP voltage-dependent K^+ channel in a lipid bilayer. *Science 306*, 491−495.

(17) Long, S. B., Campbell, E. B., and Mackinnon, R. (2005) Voltage sensor of Kv1.2: Structural basis of electromechanical coupling. *Science 309*, 903−908.

(18) Chakrapani, S., Sompornpisut, P., Intharathep, P., Roux, B., and Perozo, E. (2010) The activated state of a sodium channel voltage sensor in a membrane environment. *Proc. Natl. Acad. Sci. U.S.A. 107*, 5435−5440.

(19) Nurani, G., Radford, M., Charalambous, K., O'Reilly, A. O., Cronin, N. B., Haque, S., and Wallace, B. A. (2008) Tetrameric bacterial sodium channels: Characterization of structure, stability, and drug binding. *Biochemistry 47*, 8114−8121.

(20) Shafrir, Y., Durell, S. R., and Guy, H. R. (2008) Models of voltage-dependent conformational changes in NaChBac channels. *Biophys. J. 95*, 3663−3676.

(21) Blanchet, J., Pilote, S., and Chahine, M. (2007) Acidic residues on the voltage-sensor domain determine the activation of the NaChBac sodium channel. *Biophys. J. 92*, 3513−3523.

(22) Zhao, Y., Yarov-Yarovoy, V., Scheuer, T., and Catterall, W. A. (2004) A gating hinge in Na^+ channels: A molecular switch for electrical signaling. *Neuron 41*, 859−865.

(23) Guy, H. R., and Seetharamulu, P. (1986) Molecular model of the action potential sodium channel. *Proc. Natl. Acad. Sci. U.S.A. 83*, 508−512.

(24) Lipkind, G. M., and Fozzard, H. A. (2000) KcsA crystal structure as framework for a molecular model of the Na channel pore. *Biochemistry 39*, 8161−8170.

(25) Tikhonov, D. B., and Zhorov, B. S. (2005) Modeling P-loops domain of sodium channel: Homology with potassium channels and interaction with ligands. *Biophys. J. 88*, 184−197.

(26) MacKinnon, R., and Doyle, D. A. (1997) Prokaryotes offer hope for potassium channel structural studies. *Nat. Struct. Biol. 4*, 877−879.

(27) Ren, D., Navarro, B., Xu, H., Yue, L., Shi, Q., and Clapham, D. E. (2001) A prokaryotic voltage-gated sodium channel. *Science 294*, 2372−2375.

(28) Irie, K., Kitagawa, K., Nagura, H., Imai, T., Shimomura, T., and Fujiyoshi, Y. (2010) Comparative study of the gating motif and C-type inactivation in prokaryotic voltage-gated sodium channels. *J. Biol. Chem. 285*, 3685−3694.

(29) Koishi, R., Xu, H., Ren, D., Navarro, B., Spiller, B. W., Shi, Q., and Clapham, D. E. (2004) A superfamily of voltage-gated sodium channels in bacteria. *J. Biol. Chem. 279*, 9532−9538.

(30) Ito, M., Xu, H., Guffanti, A. A., Wei, Y., Zvi, L., Clapham, D. E., and Krulwich, T. A. (2004) The voltage-gated Na^+ channel NaVBP has a role in motility, chemotaxis, and pH homeostasis of an alkaliphilic *Bacillus*. *Proc. Natl. Acad. Sci. U.S.A. 101*, 10566−10571.

(31) Powl, A. M., O'Reilly, A. O., Miles, A. J., and Wallace, B. A. (2010) Synchrotron radiation circular dichroism spectroscopy-defined structure of the C-terminal domain of NaChBac and its role in channel assembly. *Proc. Natl. Acad. Sci. U.S.A. 107*, 14064−14069.

(32) McCusker, E. C., D'Avanzo, N., Nichols, C. G., and Wallace, B. A. (2011) Simplified bacterial "pore" provides insight into the assembly, stability and structure of sodium channels. *J. Biol. Chem. 286*, 16386−16391.

(33) Charalambous, K., O'Reilly, A. O., Bullough, P. A., and Wallace, B. A. (2009) Thermal and chemical unfolding and refolding of a eukaryotic sodium channel. *Biochim. Biophys. Acta 1788*, 1279−1286.

(34) Richardson, J., Blunck, R., Ge, P., Selvin, P. R., Bezanilla, F., Papazian, D. M., and Correa, A. M. (2006) Distance measurements reveal a common topology of prokaryotic voltage-gated ion channels in the lipid bilayer. *Proc. Natl. Acad. Sci. U.S.A. 103*, 15865−15870.

(35) O'Reilly, A. O., Charalambous, K., Nurani, G., Powl, A. M., and Wallace, B. A. (2008) G219S mutagenesis as a means of stabilizing conformational flexibility in the bacterial sodium channel NaChBac. *Mol. Membr. Biol. 25*, 670−676.

(36) Studer, A., Demarche, S., Langenegger, D., and Tiefenauer, L. (2011) Integration and recording of a reconstituted voltage-gated sodium channel in planar lipid bilayers. *Biosens. Bioelectron. 26*, 1924−1928.

(37) Kuzmenkin, A., Bezanilla, F., and Correa, A. M. (2004) Gating of the bacterial sodium channel, NaChBac: Voltage-dependent charge movement and gating currents. *J. Gen. Physiol. 124*, 349−356.

(38) Chahine, M., Pilote, S., Pouliot, V., Takami, H., and Sato, C. (2004) Role of arginine residues on the S4 segment of the *Bacillus halodurans* Na^+ channel in voltage-sensing. *J. Membr. Biol. 201*, 9−24.

(39) Abdul-Gader, A., Miles, A. J., and Wallace, B. A. (2011) A reference dataset for the analyses of membrane protein secondary structures and transmembrane residues using circular dichroism spectroscopy. *Bioinformatics 27*, 1630−1636.

(40) Mio, K., Mio, M., Arisaka, F., Sato, M., and Sato, C. (2010) The C-terminal coiled-coil of the bacterial voltage-gated sodium channel NaChBac is not essential for tetramer formation, but stabilizes subunit-to-subunit interactions. *Prog. Biophys. Mol. Biol. 103*, 111−121.

(41) Mitrovic, N., George, A. L., and Horn, R. (2000) Role of domain 4 in sodium channel slow inactivation. *J. Gen. Physiol. 115*, 707−717.

(42) Stuhmer, W., Conti, F., Suzuki, H., Wang, X. D., Noda, M., Yahagi, N., Kubo, H., and Numa, S. (1989) Structural parts involved in activation and inactivation of the sodium channel. *Nature 339*, 597−603.

(43) West, J. W., Patton, D. E., Scheuer, T., Wang, Y. L., Goldin, A. L., and Catterall, W. A. (1992) A cluster of hydrophobic amino acid residues required for fast Na^+-channel inactivation. *Proc. Natl. Acad. Sci. U.S.A. 89*, 10910−10914.

(44) DeCaen, P. G., Yarov-Yarovoy, V., Zhao, Y., Scheuer, T., and Catterall, W. A. (2008) Disulfide locking a sodium channel voltage sensor reveals ion pair formation during activation. *Proc. Natl. Acad. Sci. U.S.A. 105*, 15142−15147.

(45) Shimomura, M., Irie, K., Nagura, H., Imai, T., and Fujiyoshi, Y. (2011) Arrangement and mobility of the voltage sensor domain in prokaryotic voltage-gated sodium channels. *J. Biol. Chem. 286*, 7409−7417.

(46) Payandeh, J., Scheuer, T., Zheng, N., and Catterall, W. A. (2011) The crystal structure of a voltage-gated sodium channel. *Nature 475*, 353−358.

(47) Murata, Y., Iwasaki, H., Sasaki, M., Inaba, K., and Okamura, Y. (2005) Phosphoinositide phosphatase activity coupled to an intrinsic voltage sensor. *Nature 435*, 1239−1243.

(48) Sasaki, M., Takagi, M., and Okamura, Y. (2006) A voltage sensor-domain protein is a voltage-gated proton channel. *Science 312*, 589−592.

(49) Lu, Z., Klem, A. M., and Ramu, Y. (2001) Ion conduction pore is conserved among potassium channels. *Nature 413*, 809−813.

(50) Chakrapani, S., Cuello, L. G., Cortes, D. M., and Perozo, E. (2008) Structural dynamics of an isolated voltage-sensor domain in a lipid bilayer. *Structure 16*, 398−409.

(51) Aggarwal, S. K., and MacKinnon, R. (1996) Contribution of the S4 segment to gating charge in the Shaker K^+ channel. *Neuron 16*, 1169−1177.

(52) Seoh, S. A., Sigg, D., Papazian, D. M., and Bezanilla, F. (1996) Voltage-sensing residues in the S2 and S4 segments of the Shaker K^+ channel. *Neuron 16*, 1159−1167.

(53) Armstrong, C. M., and Hille, B. (1998) Voltage-gated ion channels and electrical excitability. *Neuron 20*, 371−380.

(54) Heinemann, S. H., Terlau, H., Stuhmer, W., Imoto, K., and Numa, S. (1992) Calcium channel characteristics conferred on the sodium channel by single mutations. *Nature 356*, 441−443.

(55) Wu, X. S., Edwards, H. D., and Sather, W. A. (2000) Side chain orientation in the selectivity filter of a voltage-gated Ca^{2+} channel. *J. Biol. Chem. 275*, 31778−31785.

(56) Yue, L., Navarro, B., Ren, D., Ramos, A., and Clapham, D. E. (2002) The cation selectivity filter of the bacterial sodium channel, NaChBac. *J. Gen. Physiol. 120*, 845−853.

(57) Motoike, H. K., Liu, H., Glaaser, I. W., Yang, A. S., Tateyama, M., and Kass, R. S. (2004) The Na^+ channel inactivation gate is a molecular complex: A novel role of the COOH-terminal domain. *J. Gen. Physiol. 123*, 155−165.

(58) Abriel, H., Wehrens, X. H., Benhorin, J., Kerem, B., and Kass, R. S. (2000) Molecular pharmacology of the sodium channel mutation D1790G linked to the long-QT syndrome. *Circulation 102*, 921−925.

(59) Clancy, C. E., and Rudy, Y. (2002) Na^+ channel mutation that causes both Brugada and long-QT syndrome phenotypes: A simulation study of mechanism. *Circulation 105*, 1208−1213.

(60) Fujiwara, T., Sugawara, T., Mazaki-Miyazaki, E., Takahashi, Y., Fukushima, K., Watanabe, M., Hara, K., Morikawa, T., Yagi, K., Yamakawa, K., and Inoue, Y. (2003) Mutations of sodium channel α subunit type 1 (SCN1A) in intractable childhood epilepsies with frequent generalized tonic-clonic seizures. *Brain 126*, 531−546.

(61) Chagot, B., Potet, F., Balser, J. R., and Chazin, W. J. (2009) Solution NMR structure of the C-terminal EF-hand domain of human cardiac sodium channel NaV1.5. *J. Biol. Chem. 284*, 6436−6445.

(62) Miloushev, V. Z., Levine, J. A., Arbing, M. A., Hunt, J. F., Pitt, G. S., and Palmer, A. G. III (2009) Solution structure of the $Na_V1.2$ C-terminal EF-hand domain. *J. Biol. Chem. 284*, 6446−6454.

(63) Jenke, M., Sanchez, A., Monje, F., Stuhmer, W., Weseloh, R. M., and Pardo, L. A. (2003) C-terminal domains implicated in the functional surface expression of potassium channels. *EMBO J. 22*, 395−403.

(64) Tinker, A., Jan, Y. N., and Jan, L. Y. (1996) Regions responsible for the assembly of inwardly rectifying potassium channels. *Cell 87*, 857−868.

(65) Demo, S. D., and Yellen, G. (1991) The inactivation gate of the Shaker K^+ channel behaves like an open-channel blocker. *Neuron 7*, 743−753.

(66) Pavlov, E., Bladen, C., Winkfein, R., Diao, C., Dhaliwal, P., and French, R. J. (2005) The pore, not cytoplasmic domains, underlies inactivation in a prokaryotic sodium channel. *Biophys. J. 89*, 232−242.

(67) Zhao, Y., Scheuer, T., and Catterall, W. A. (2004) Reversed voltage-dependent gating of a bacterial sodium channel with proline substitutions in the S6 transmembrane segment. *Proc. Natl. Acad. Sci. U.S.A. 101*, 17873−17878.

(68) Zhou, Y. F., Morais-Cabral, J. H., Kaufman, A., and MacKinnon, R. (2001) Chemistry of ion coordination and hydration revealed by a K^+ channel-Fab complex at 2.0 angstrom resolution. *Nature 414*, 43−48.

(69) Cestele, S., and Catterall, W. A. (2000) Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie 82*, 883−892.

(70) Catterall, W. A., Cestele, S., Yarov-Yarovoy, V., Yu, F. H., Konoki, K., and Scheuer, T. (2007) Voltage-gated ion channels and gating modifier toxins. *Toxicon 49*, 124−141.

(71) Catterall, W. A. (1992) Cellular and molecular biology of voltage-gated sodium channels. *Physiol. Rev. 72*, S15−S48.

(72) Ouyang, W., Jih, T. Y., Zhang, T. T., Correa, A. M., and Hemmings, H. C. (2007) Isoflurane inhibits NaChBac, a prokaryotic voltage-gated sodium channel. *J. Pharmacol. Exp. Ther. 322*, 1076−1083.

(73) Ellinor, P. T., Yang, J., Sather, W. A., Zhang, J. F., and Tsien, R. W. (1995) Ca^{2+} channel selectivity at a single locus for high affinity Ca^{2+} interactions. *Neuron 15*, 1121−1132.

(74) Hockerman, G. H., Peterson, B. Z., Johnson, B. D., and Catterall, W. A. (1997) Molecular determinants of drug binding and action on L-type calcium channels. *Annu. Rev. Pharmacol. Toxicol. 37*, 361−396.

(75) Hille, B. (1989) The Sharpey-Schafer Lecture. Ionic channels: Evolutionary origins and modern roles. *Q. J. Exp. Physiol. Cogn. Med. Sci. 74*, 785−804.

(76) Gilly, W. F., Gillette, R., and McFarlane, M. (1997) Fast and slow activation kinetics of voltage-gated sodium channels in molluscan neurons. *J. Neurophysiol. 77*, 2373−2384.

(77) Jarvis, M. F., Honore, P., Shieh, C. C., Chapman, M., Joshi, S., Zhang, X. F., Kort, M., Carroll, W., Marron, B., Atkinson, R., Thomas, J., Liu, D., Krambis, M., Liu, Y., McGaraughty, S., Chu, K., Roeloffs, R., Zhong, C. M., Mikusa, J. P., Hernandez, G., Gauvin, D., Wade, C., Zhu, C., Pai, M., Scanio, M., Shi, L., Drizin, I., Gregg, R., Matulenko, M., Hakeem, A., Grosst, M., Johnson, M., Marsh, K., Wagoner, P. K., Sullivan, J. P., Faltynek, C. R., and Krafte, D. S. (2007) A-803467, a potent and selective $Na_v1.8$ sodium channel blocker, attenuates neuropathic and inflammatory pain in the rat. *Proc. Natl. Acad. Sci. U.S.A. 104*, 8520−8525.

(78) Nelson, R.D., Kuan, G., Saier, M. H. Jr., and Montal, M. (1999) Modular assembly of voltage-gated channel proteins: A sequence analysis and phylogenetic study. *J. Mol. Microbiol. Biotechnol. 1*, 281−287.

(79) Clapham, D. E., and Garbers, D. L. (2005) International Union of Pharmacology. L. Nomenclature and structure-function relationships of CatSper and two-pore channels. *Pharm. Rev. 57*, 451−454.

(80) Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Clustal-W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res. 22*, 4673−4680.

(81) Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. L. (2001) Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J. Mol. Biol. 305*, 567−580.