Molecular Determinants of Na\(^+\) Channel Function in the Extracellular Domain of the \(\beta 1\) Subunit*

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The rat brain voltage-gated Na\(^+\) channel is composed of three glycoprotein subunits: the pore-forming \(\alpha\) subunit and two auxiliary subunits, \(\beta 1\) and \(\beta 2\), which contain immunoglobulin (Ig)-like folds in their extracellular domains. When expressed in Xenopus oocytes, \(\beta 1\) modulates the gating properties of the channel-forming type IIA \(\alpha\) subunit, resulting in an acceleration of inactivation. We have used a combination of deletion, alanine-scanning, site-directed, and chimeric mutagenesis strategies to examine the importance of different structural features of the \(\beta 1\) subunit in the modulation of \(\alpha_{IIA}\) function, with an emphasis on the extracellular domain. Deletion analysis revealed that the extracellular domain is required for function, but the intracellular domain is not. The mutation of four putative sites of N-linked glycosylation showed that they are not required for \(\beta 1\) function. Mutations of hydrophobic residues in the core \(\beta\) sheets of the Ig fold disrupted \(\beta 1\) function, whereas substitution of amino acid residues in connecting segments had no effect. Mutations of acidic residues in the \(\alpha/\alpha'\) strand of the Ig fold reduced the effectiveness of the \(\beta 1\) subunit in modulating the rate of inactivation but did not significantly affect the association of the mutant \(\beta 1\) subunit with the \(\alpha_{IIA}\) subunit or its effect on recovery from inactivation. Our data suggest that the Ig fold of the \(\beta 1\) extracellular domain serves as a scaffold that presents the charged residues of the \(\alpha/\alpha'\) strands for interaction with the pore-forming \(\alpha\) subunit.

The rat brain voltage-gated sodium (Na\(^+\)) channel is composed of three glycoprotein subunits: the pore-forming \(\alpha\) subunit with a relative molecular mass of 260 kDa, and two auxiliary subunits, \(\beta 1\) (36 kDa) and \(\beta 2\) (33 kDa) (for review, see Refs. 1 and 2). The \(\alpha\) subunit has four internally homologous domains, each containing six potential transmembrane-spanning regions and a pore-forming loop (3). The \(\beta 1\) and \(\beta 2\) subunits from rat brain are not closely related in terms of amino acid sequence, but each contains a single membrane-spanning segment that separates a large NH\(_2\)-terminal extracellular domain from a smaller COOH-terminal intracellular domain (4, 5). In addition, the extracellular domains of the \(\beta 1\) and \(\beta 2\) subunits have sequences similar to those of proteins of the immunoglobulin (Ig)\(^1\) superfamily and are proposed to contain an Ig-like motif (5, 6). The \(\beta 2\) subunit is linked covalently to the \(\alpha\) subunit by disulfide bonds, whereas the \(\beta 1\) subunit associates with the \(\alpha\) subunit in a noncovalent manner (7). Biochemical analyses of detergent-solubilized rat brain Na\(^+\) channels suggest that both ionic and hydrophobic interactions are important in association of \(\beta 1\) with the \(\alpha/\beta 2\) complex (7).

Although the auxiliary subunits are not required for the formation of functional Na\(^+\) channels (8, 9), coexpression of the \(\beta 1\) subunit with the rat brain type IIA \(\alpha\) subunit in Xenopus oocytes increases the proportion of Na\(^+\) channels that function in a fast gating mode (4, 10). Na\(^+\) channel inactivation is accelerated 5-fold, the voltage dependence of inactivation is shifted in the negative direction, and a larger fraction of channels recovers rapidly from inactivation. In addition, the amplitude of peak Na\(^+\) current is increased, consistent with an increase in channel expression. In Chinese hamster lung cells, coexpression of \(\beta 1\) with the type IIA \(\alpha\) subunit results in hyperpolarizing shifts in the voltage dependence of Na\(^+\) channel activation and inactivation and increased channel expression (11). The \(\beta 1\) subunit modulates the gating and expression of a variety of Na\(^+\) channel \(\alpha\) subunits (10, 12–14), suggesting that the interaction domains of these two proteins are well conserved.

In the present study we examine the structural features of the \(\beta 1\) subunit which are required for efficient modulation of rat brain type IIA \(\alpha\) subunits. We demonstrate that the intracellular domain of \(\beta 1\) is unnecessary and that the extracellular domain is essential for expression and function of the \(\alpha/\beta 1\) complex in Xenopus oocytes. A combinatorial approach using deletion mutagenesis, alanine-scanning mutagenesis, and chimeric protein analyses supports the hypothesis that the extracellular domain forms an Ig fold that is essential for \(\beta 1\) expression and function. Through analysis of these mutants we have implicated the \(\alpha/\alpha'\) strand of the Ig fold in the \(\beta 1\) extracellular domain in interactions between \(\beta 1\) and \(\alpha\) subunits. This region is localized on a presumed surface-exposed edge of the Ig fold motif. While our experiments were in progress, deletion and chimeric mutagenesis studies of the modulation of skeletal muscle Na\(^+\) channels by \(\beta 1\) subunits were reported (15, 16). These results are compared with our data under “Discussion.”

EXPERIMENTAL PROCEDURES

Plasmid Construction—The deletion of the \(\beta 1\) intracellular domain (Fig. 1A, \(\Delta 1\)) and the large internal deletion of the \(\beta 1\) extracellular domain (Fig. 1A, \(\Delta 2\)) were constructed in the Bluescript pSK\(^+\) phagemid (Stratagene, La Jolla, CA). Subsequent analyses of wild-type and mutant \(\beta 1\) subunits were performed in the pBLSK\(^-\) vector in which the entire coding sequence of the rat brain \(\beta 1\) subunit and 3\'-untranslated

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* The abbreviations used are: Ig, immunoglobulin; \(F_c\) and \(F_h\), fraction of channel in fast and slow gating mode, respectively; \(\gamma_c\) and \(\tau_c\), time constants in fast and slow mode, respectively; P50, protein zero.
region, including polyadenylation sequences, were cloned into Bluescript plasmid pSK+ (Stratagene). The pβ1.SK and pβ1.SK+ vectors were constructed in a manner that eliminated a portion of the β1' untranslated region which had been found previously to interfere with expression (10).

**Mutagenesis**—The deletion of the β1 intracellular domain (Fig. 1A, Δ1) was accomplished via polymerase chain reaction mutagenesis by the substitution of a termination signal (TAA) for the Ile1546 codon. The large internal deletion (Fig. 1A, Δ2) of the β1 extracellular domain was constructed by an in-frame removal of a BstYI fragment. Two sets of three nested deletions in the cDNA encoding the extracellular domain of the β1 subunit were produced by oligonucleotide-directed "loop out" deletion mutagenesis (Fig. 1A, Δ3–Δ8). The NH2-terminal signal sequence was maintained in all deletion mutants to facilitate proper membrane insertion and post-translational processing. Single stranded pβ1.SK+ DNA was prepared by helper phage infection and served as the mutagenesis template. Deletion mutagenesis reactions were performed using the Oligonucleotide-directed In Vitro Mutagenesis System Version 2 or Sculptor104 protocols (Amersham Corp.). Alix substitutions, ΔAΔ strand change neutralizations, and β1PΔ chimeras were constructed in β1.M13Mp18 by oligonucleotide-directed mutagenesis. Mutagenesis reactions were according to the protocol of Kunkel et al. (17). Mutants were identified by DNA sequencing or by incorporation of a silent restriction endonuclease site and then subcloned into the pβ1.SK+ phagemid. Sequences of the mutant constructs were determined by automated sequence analysis performed by the DNA Core of the Molecular Pharmacology Facility, Department of Pharmacology, University of Washington.

**In Vitro Transcription of RNA and Expression of Na+ Channels in Xenopus Oocytes**—RNA transcripts were obtained using the mMessage mMachine in vitro transcription protocol (Ambion, Inc., Austin, TX). Transcripts were purified by sedimentation through columns of Sephadex G-50. The rat α1a, subunit was transcribed using T7 RNA polymerase from plasmid pVA2580 (18), which had been linearized with ClaI. The β1Δ1 construct was linearized with HindIII, and RNA was transcribed with T7 RNA polymerase. The β1Δ2 construct was linearized with EcoRI, and RNA was transcribed with T7 RNA polymerase. Wild-type and mutant β1 constructs in pβ1.SK+ were linearized with HindIII, and RNA was transcribed using T3 RNA polymerase. *N. laevis* oocytes were harvested, defolliculated with collagenase, and maintained as described (19). Oocytes were injected with a 50-nl volume of an RNA mixture containing 0.5–1.25 ng of α1a transcript and a 5–10-fold excess of mutant β1 subunit transcript, unless otherwise stated in the figure legends. Competition experiments were employed to determine if the ability to associate with the α1a subunit was retained in mutant β1 subunits that showed decreased modulatory β1 function. In these experiments, α1a, and wild-type β1 subunits were injected in combination with the mutant β1 subunit. If the mutant β1 subunit is capable of binding to the α1a subunit, less than maximum β1 function should be observed when analyzed by electrophysiological methods. Alternatively, wild-type β1 function in the expressing mutant β1 oocytes would indicate an inability of the mutant β1 to bind to the α1a subunit. In all experiments, oocytes injected with only α1a transcript or α1a, co-injected with wild-type β1 RNA served as controls.

**Electrophysiological Recording**—After 48 h at 20 °C, expressed Na+ channels were examined at room temperature by two-electrode voltage clamp (Dagan CA1, Minneapolis, MN; see Ref. 19). Voltage pulses were applied, and data were recorded using the Basic-Fastlab data acquisition system (Indec Systems, Capitola, CA). Linear capacity currents were canceled using the internal voltage clamp circuitry. Residual linear currents were subtracted using the P4 procedure (20). Signals were low pass filtered at 2 kHz. The amplitude of expressed Na+ currents was typically 1–10 μA. The bath was perfused continuously with Frog Ringer containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 10 mM HEPES, pH 7.2. Na+ currents were elicited by step depolarizations to 0 mV from a holding potential of -90 mV. Prepulses of 100-ms duration to various voltages, followed by a test pulse to 0 mV, were used to examine the voltage dependence of steady-state fast inactivation of Na+ channels. Na+ channel current-voltage relationships were determined from peak currents elicited by 30-ms steps to various test voltages from a holding potential of -90 mV. Recovery from inactivation was examined by applying a 15-ms conditioning pulse to 0 mV from a holding potential of -90 mV followed by a recovery interval of variable duration and a test pulse to 0 mV.

**Statistical Analysis of Electrophysiological Data**—Representative normalized current traces obtained from oocytes co-injected with α1a and mutant β1 RNA transcripts were plotted with normalized averaged current traces from a contemporaneous pool of control oocytes containing only α1a transcript or with wild-type β1 RNA transcripts. The standard deviation of the control oocyte traces was calculated and is plotted as dotted lines in each figure. Additional statistical analyses were performed on current traces obtained from oocytes co-injected with α1a and β1(E4Q/D6N/E8Q). To determine the mean fractions of Na+ channels in the slow and fast gating mode (Ff and Fs) and the corresponding time constants (τs and τf), the decay phase of Na+ current traces was fit to a single exponential or a sum of two exponentials. Current traces obtained from oocytes injected with only the α1a transcript were well fit by a single exponential. Two exponentials were used to fit the traces obtained from oocytes co-injected with transcripts encoding α1a and wild-type β1 or with α1a and β1(E4Q/D6N/E8Q) transcripts. Through these analyses it was determined that the time constants obtained from current traces from oocytes expressing the α1a, and β1(E4Q/D6N/E8Q) transcripts (τs = 4.58 ± 1.42 ms, τf = 0.917 ± 0.474 ms, n = 6) did not vary significantly from τs values for α1a control cells (τs = 4.74 ± 0.62 ms, n = 6) or τf values obtained from control oocytes that coexpressed the α1a, and wild-type β1 subunits (τf = 0.892 ± 0.156 ms, n = 7). Student’s t test p values for the comparison of the time constants for α1a and β1(E4Q/D6N/E8Q) with control time constants were 0.90 for τs and 0.004 for τf, respectively, confirming that the differences between these values were not significant. To obtain accurate values for the fraction of oocytes gating in the fast mode (Ff) and slow mode (Fs), current traces from oocytes expressing α1a alone, α1a and wild-type β1, or α1a, and β1(E4Q/D6N/E8Q), and from oocytes expressing combinations of these subunits in competition experiments were refit using mean values of...
the control cell \( \tau \) and \( \tau \) values defined above. The values for \( F_r \) and \( F \) obtained from these fits were compared, and the significance of the observed differences was determined by \( t \) test analyses.

Data obtained from experiments measuring recovery from inactivation were normalized and fit with either one or two exponentials, as described in the figure legends. The \( F_r \), \( F \), and corresponding \( \tau \) values of channel recovery from inactivation were determined from the fits.

RESULTS

The Intracellular Domain of the Rat Brain \( \beta_1 \) Subunit Is Not Required for Modulation of a Subunit Function

—An important step in understanding the mechanism by which the \( \beta_1 \) subunit modulates a subunit function is to determine which domains of \( \beta_1 \) participate in a-\( \beta_1 \) interactions and subsequent modulation of channel gating. To test the role of the intracellular domain, a truncated rat brain \( \beta_1 \) subunit, lacking the intracellular domain, was constructed by the insertion of a stop codon at the position normally encoding Ile\textsubscript{166} (Fig. 1A, \( \Delta 1 \)). Lys residues at positions 164 and 165, immediately following the proposed transmembrane segment, were retained to maintain proper membrane orientation. Xenopus oocytes were prepared and co-injected with \textit{in vitro} transcribed RNA encoding the adult rat type IIA \( \alpha \) subunit alone or in combination with wild-type or mutant \( \beta_1 \) subunits. Na\textsuperscript{+} currents were recorded from the injected oocytes at room temperature using a two-electrode voltage clamp. Na\textsuperscript{+} channels containing only \( \alpha_{IA} \) subunits inactivated slowly (Fig. 1B, trace a), whereas those containing \( \alpha_{IA} \) and wild-type \( \beta_1 \) inactivated more rapidly (Fig. 1B, trace b). Coinjection of RNA encoding the truncated \( \beta_1 \) subunit and \( \alpha_{IA} \) subunit in Xenopus oocytes resulted in rapidly inactivating Na\textsuperscript{+} currents that were similar to those seen when the \( \alpha_{IA} \) was coexpressed with the wild-type \( \beta_1 \) subunit (Fig. 1B, trace c).

Because both the wild-type and truncated \( \beta_1 \) subunits induced predominantly fast inactivating channels, the intracellular domain of the \( \beta_1 \) subunit is not necessary for either the association of Na\textsuperscript{+} channel \( \alpha \) and \( \beta_1 \) subunits or for functional modulation of the \( \alpha \) subunit by the \( \beta_1 \) subunit.

Deletion Analysis of the Extracellular Domain of \( \beta_1 \)

The importance of the extracellular domain was investigated further by a series of deletion mutations (Fig. 1A). An internal deletion of the extracellular domain was constructed which removed amino acids Ile\textsubscript{51} through Lys\textsubscript{122} (Fig. 1A, \( \Delta 2 \)). When the \( \alpha \) subunit was coexpressed with the Ile\textsubscript{51}-Lys\textsubscript{122} deletion mutant in Xenopus oocytes, the resulting Na\textsuperscript{+} currents were similar to those of channels composed of \( \alpha \) subunits alone (data not shown), indicating that the large internal deletion of the extracellular domain prevented expression and/or function of the \( \beta_1 \) subunit. To investigate further the importance of the extracellular domain, nested deletions in the cDNA encoding the extracellular domain of the \( \beta_1 \) subunit were produced by oligonucleotide-directed loop out mutagenesis (Fig. 1A, \( \Delta 3-\Delta 8 \)). The time courses of Na\textsuperscript{+} currents resulting from coexpression of \( \alpha \) with the smallest of these \( \beta_1 \) deletion mutants (\( \Delta 3 \), Gly\textsuperscript{2}-Phe\textsuperscript{45}, Fig. 1B, trace d; \( \Delta 6 \) Val\textsuperscript{116}-Ser\textsuperscript{140}, Fig. 1B, trace e) indicated that they decayed at a rate similar to channels consisting of only the \( \alpha \) subunit. Similar results were obtained for each electrophysiological parameter tested for these and the remaining deletion mutants. In addition, \( \beta_1 \) subunits harboring either \( \Delta 3 \) (Gly\textsuperscript{2}-Phe\textsuperscript{45}) or \( \Delta 6 \) (Val\textsuperscript{116}-Ser\textsuperscript{140}) were unable to compete with the wild-type \( \beta_1 \) subunit for binding to \( \alpha_{IA} \) (data not shown; for further description of the competition experiments, see “Experimental Procedures”). These results highlight the requirement for the integrity of the extracellular domain for function of the \( \beta_1 \) subunit.

Analysis of N-Linked Glycosylation Sites

The extracellular domain of the \( \beta_1 \) subunit contains four potential sites for N-linked glycosylation at Asn\textsuperscript{74}, Asn\textsuperscript{91}, Asn\textsuperscript{95}, and Asn\textsuperscript{116} (4), and biochemical experiments show that \( \beta_1 \) subunits have at least three N-linked carbohydrate chains (21). To investigate the importance of these sites in the modulation of Na\textsuperscript{+} channel gating by the \( \beta_1 \) subunit, the Asn residues of all four sites were mutated individually to Gln. These mutant subunits showed no significant loss of \( \beta_1 \) function, as assessed in the Xenopus oocyte expression system (Fig. 2). The adjacent glycosylation sites at Asn\textsuperscript{91} and Asn\textsuperscript{95} were also altered simultaneously. The resulting \( \beta_1 \) double mutant behaved normally, like the single site mutants (Fig. 2). These results show that none of the N-linked glycosylation sites is required for expression and function of the \( \beta_1 \) subunits.

The Extracellular Domain of \( \beta_1 \) Is Predicted to Form an Ig Fold

—Data-base searches found sequence similarities between the \( \beta_1 \) extracellular domain and members of the Ig fold superfamily (5, 6). The resemblance is closest with the V-like family of Ig fold proteins, which contain domains resembling the variable regions of antibodies and include many cell adhesion molecules and cell recognition proteins (22, 23). The \( \beta_1 \) extracellular domain has the highest degree of similarity to that of myelin protein protein (\( P_\beta \)), with 36% identity and 49% similarity over a 70-amino acid segment (5). \( P_\beta \) is the major protein in peripheral nerve myelin and mediates the wrapping of myelin sheaths via homophilic interactions (23). Its structure has been studied by molecular modeling (24), and the crystal structure of the rat \( P_\beta \) extracellular domain (\( P_{\text{ex}} \)) was determined recently at 1.9 Å resolution (25). Both analyses show that \( P_{\text{ex}} \) is folded as a V-like Ig domain.

Alignment of the amino acid sequences of the \( \beta_1 \) subunit and \( P_{\text{ex}} \) (Fig. 3A) reveals that both conform closely to a consensus sequence for V-like Ig folds, like many cell adhesion molecules (23). The \( \beta \) strands A–G that form the Ig fold are highlighted in Fig. 3A and are illustrated in their three-dimensional organization in Fig. 3B. In this alignment Cys\textsuperscript{21} of the \( \beta_1 \) subunit is shown in register with the Cys residues that form the disulfide bond in the Ig fold of \( P_\beta \) rather than Cys\textsuperscript{24} as in our previous alignment (6), because this fixes the new x-ray structure (25) and the new V-like consensus (23) more closely. The remaining Cys residues of the \( \beta_1 \) extracellular domain, Cys\textsuperscript{22} and Cys\textsuperscript{24}, are in close proximity in our Ig fold model and may form an additional disulfide bridge between the beginning of the A strand and the end of the B strand. In addition to the invariant Cys residues in \( \beta \) strands B and F, other important Ig-fold residues, such as the Gly preceding strand B, the Trp in strand C, and the Arg in strand D, are aligned precisely in the folding model of \( \beta_1 \) (Fig. 3B), and many other requirements for a V-like Ig fold are fulfilled (Fig. 3A). Myelin protein \( P_\beta \) therefore provides a molecular template for analysis of the structure of the \( \beta_1 \) subunit.
based on its three-dimensional structure illustrated in Fig. 3B.

Analysis of the Core $\beta$ Strands by Alanine-scanning Site-directed Mutagenesis—The Ig fold motif is a “sandwich” of two $\beta$ sheets. Side chains from both $\beta$ sheets form a hydrophobic core at the interface between the $\beta$ sheets by interaction of the alternating hydrophobic amino acids in the primary sequence of core $\beta$ strands. The central hydrophobic core, composed primarily of $\beta$ strands B and E in one sheet and strands C, C', and F in the other, is crucial for the maintenance of the Ig fold (22). The central core of the Ig fold serves as a scaffold for the presentation of sites involved in molecular recognition, cell adhesion, and ligand binding.

Alanine-scanning mutagenesis (26) was employed to test whether the central core of the Ig fold is necessary for expression and function of the $\beta1$ subunits. Ala was chosen as the replacement amino acid because it has a small side chain and does not introduce electrostatic effects or alter the main chain conformation. Although Ala is a strong $\alpha$-helix former, it shows a significant propensity for $\beta$ sheet formation and is found in both buried and exposed positions (27–30). Replacement of hydrophobic amino acid residues in the core of proteins with Ala has been shown not to cause global structural change (31). However, in the case of an Ig fold, substitution of Ala for multiple hydrophobic amino acids in a $\beta$ strand that contributes to the hydrophobic core of the Ig fold should disrupt the tertiary structure of the domain.

The alanine-scanning mutagenesis survey of proposed $\beta$ strands in the $\beta1$ extracellular domain is summarized in Fig. 4A. The substitution of Ala for multiple large, hydrophobic amino acids in the mutants AS-1, AS-2, AS-3, AS-5, and AS-6 resulted in slowly inactivating $\text{Na}^+$ currents resembling those exhibited by the $\alpha$ subunit in the absence of $\beta1$ (Fig. 4B, traced). A Trp residue at the position of Trp38, combined with the F35A and F40A mutations most likely resulted in a perturbation of the Ig fold hydrophobic core. A similar disruption of hydrophobic interactions in the central core of the Ig fold is likely to be responsible for the effects of mutations AS-1, AS-3, AS-5, and AS-6. None of $\beta1$ subunits harboring the deleterious alanine cluster mutations was capable of competing with the wild-type $\beta1$ subunit for binding to $\alpha1\text{IIA}$ (data not shown), which suggests that the alanine substitutions either dramatically inhibited $\alpha$-$\beta1$ interactions or prevented $\beta1$ folding and cell surface expression.

In contrast to the effects of mutations in these central core $\beta$
strands, the mutation of three hydrophobic amino acid residues in the D strand in the AS-4 mutant (Fig. 4C, trace f) had no effect on expression and function of β1 subunits. A similar result was observed for the AS-8 mutant, which targeted the peripherally located G strand (Fig. 4C, trace i). Our findings show that perturbation of the central hydrophobic core structure of the Ig fold prevents β1 function whereas similar mutations in peripheral β strands have little effect. Thus, it is likely that hydrophobic amino acids in the central segments contribute to the stability of the core of the Ig fold and that disruption of the central core of the Ig fold prevents β1 expression and function. These results are consistent with the hypothesis that the Ig fold forms a scaffold to present interaction site(s) to the α subunit for specific association.

**Mutations of Loops Connecting the β Strands of the Ig Fold of the β1 Subunit**—In Ig fold proteins such as antibody variable domains and cell adhesion molecules, the sites of interaction with ligands are generally confined to the hypervariable segments connecting the core β strands (22, 23). Many of the loop regions of the β1 extracellular domain do not contain large hydrophobic amino acids and therefore are not investigated easily by alanine-scanning mutagenesis. To investigate the importance of these loops for β1 function, chimeric proteins were constructed in which the loops connecting the strands of the rat brain Na⁺ channel β1 subunit were replaced with the corresponding amino acids of myelin P₀ protein. Although predicted to be topologically similar to β1, the rat isoform of myelin P₀ does not modulate Na⁺ channel gating in the Xenopus oocyte expression system (data not shown), indicating that differences in specific amino acid residues within these two closely similar structures must be responsible for interaction with Na⁺ channel α subunits.

Substitutions of clusters of amino acid residues from the myelin P₀ protein into the B-C, C-C’ , C’-C”, C”-D, and D-E loops were made as diagrammed in Fig. 5A. Surprisingly, all five ββP₀ chimeras functioned like wild-type β1 (Fig. 5, B and C). In addition, because the proposed D-E loop of β1 is longer than the corresponding loop of myelin P₀, we also studied deletion and single amino acid mutations in that loop. Three amino acids, Thr79, Lys80, and Asp81 were deleted from the D-E loop, with no discernible effect on β1 function (data not shown). In addition, the simultaneous substitution of Leu²² and Leu²⁸ with Ala resulted in no detectable loss of β1 function (data not shown). Taken together, these results suggest that the amino acid residues replaced or deleted in the loop regions we examined do not contribute significantly to α-β1 interactions and that the additional length of the β1 D-E loop is not an important factor for these interactions.

**Hydrophobic Amino Acid Residues in the Juxtamembrane Region of the β1 Subunit Are Not Required for β1 Function**—The Ig fold of the β1 subunit is connected to its transmembrane domain by a juxtamembrane domain of 11 residues (Fig. 3A). We felt that this region was a good candidate for interaction with the α₁IA subunit because it is located near the membrane surface, where it could potentially interact with even the smallest extracellular loops of α₁IA. Positions Met₁₃⁵, Ile₁₃⁸, and Val₁₄⁸ in the juxtamembrane region were targeted because of the propensity of hydrophobic residues for protein interaction sites (32). Individual Ala substitutions of these amino acid positions were constructed, as well as an Ala cluster mutation (AS-9) in which all three hydrophobic amino acid residues in this segment were replaced by Ala (Fig. 4A). The cluster mutation and each of the individual Ala substitutions retained β1 function that was comparable to wild-type β1 (Fig. 6, traces c, d, e, and f). These results indicate that the hydrophobic amino acids of the β1 juxtamembrane region do not play an important role in β1 function.
Intervening amino acids are involved in intersheet hydrogen bonding, these negative charges would be arrayed on the external surface of the Ig fold. Therefore, these charged residues may be accessible for interaction with extracellular regions of the Na⁺ channel α subunit and may contribute to the ionic interactions that were found to play a large role in the stability of the purified α-β1 complex (7).

We examined the importance of the negative charges in the A and A' strands by site-directed mutagenesis. A β1 mutant was created in which three of the four negatively charged residues in the A and A' strands were neutralized (E4Q/D6N/E8Q). Care was taken to replace the native residues with amino acids of equal or better β sheet-forming propensity (30). The analysis of oocytes coinjected with αIA and β1(E4Q/D6N/E8Q) transcripts (1:1 w/w) showed that the β1 A/A' strand mutant was capable of only partial modulation of α subunit function, as assessed from the macroscopic kinetics of Na⁺ channel inactivation (Fig. 7A, compare traces a-c). The rate of activation of channels containing the A/A' strand mutant (trace c) was faster than for α subunits alone (trace a), but significantly slower than those of channels comprised of αIA and wild-type β1 (trace b). To test whether this difference was caused by poor expression of β1(E4Q/D6N/E8Q), oocytes were injected with αIA transcript plus a 10-fold excess of the β1(E4Q/D6N/E8Q) transcript. As with the lower concentration of β1(E4Q/D6N/E8Q) transcript, the rate of inactivation of the β1 A/A' strand mutant was substantially slower than that observed for cells injected with a 1:10 weight ratio of αIA to wild-type β1 (Fig. 7B, compare traces b and c). Similar results were obtained with a 50-fold excess of β1(E4Q/D6N/E8Q) injected mRNA (data not shown). In addition, the rate of inactivation for channels containing the β1 A/A' strand mutant channels was consistently slower than for channels containing wild-type β1 at test pulses from 0 mV to +20 mV (data not shown).

The differences in the effects of wild-type and mutant β1 subunits were confirmed by statistical analysis of the inactivation kinetics of channels containing the β1(E4Q/D6N/E8Q) subunit compared with control Na⁺ channels during a 30-ms test pulse to 0 mV from a holding potential of −90 mV. Oocytes expressing αIA in combination with either the wild-type or mutant β1 subunit exhibited two exponential decay components with distinct fast and slow time constants. In contrast, channels comprised of only the αIA subunit displayed only the
slower time constant. Our analysis found no statistically significant differences between the time constants of the fast and slow components of Na⁺ current decay for channels containing the β1(E4Q/D6N/E8Q) mutation and those of control channels (see “Experimental Procedures”). This is consistent with our hypothesis that the β1 subunit causes a shift in the fraction of Na⁺ channels in fast and slow gating modes without changes of the kinetics within each gating mode (14, 10). However, channels containing the A/A’ strand mutant β1 subunit exhibited a 22% reduction in the fraction of channels inactivating with the fast time constant relative to channels composed of αIIIA and wild-type β1 subunits at a 1:1 ratio of α and β1 transcripts and a 16% reduction at a 1:10 ratio of α and β1 transcripts. The mean values for Fₜ and their standard errors are illustrated as a box plot in Fig. 7C. This statistical analysis shows that these differences in the Fₜ are statistically significant.

Competition experiments were carried out to assess the ability of the β1 A/A’ strand mutant to bind to αIIIA and block the effect of wild-type β1. The β1 A/A’ mutant competed with the wild-type β1 subunit for binding to αIIIA when cojected in equal amounts or in a 10-fold excess (Fig. 7A, traces d and e), producing channels that inactivated with the intermediate rate characteristic of the mutant β1 subunit rather than the faster rate characteristic of wild-type. As in the previous experiments, the slower inactivation rate was caused by a decrease in the fraction of channels inactivating with the fast time constant (Fig. 7C).

Further evidence that the A/A’ strand β1 mutant interacts effectively with the αIIIA subunit was obtained from analysis of the recovery from inactivation of Na⁺ channels (Fig. 7D). Recovery from inactivation of Na⁺ channels containing wild-type β1 subunits was fast, with essentially complete recovery in a
single exponential time course with $\tau = 3.1 \pm 0.9$ ms ($n = 5$). In contrast, Na$^+$ channels containing only $\alpha_{IIA}$ subunits have a biphasic recovery from inactivation with a prominent fast phase having $\tau = 2.3 \pm 0.3$ ms and a slow phase having $\tau = 245 \pm 9$ ms ($n = 4$). Na$^+$ channels containing the $\beta1 A/A'$ strand mutant recovered predominantly fast, with $\tau = 3.1 \pm 1.1$ ms ($n = 4$) and were indistinguishable from channels containing the wild-type $\beta1$ subunit on the basis of recovery from inactivation (Fig. 7B). The results of experiments on recovery from inactivation show that the $\beta1 A/A'$ strand mutant binds to the $\alpha_{IIA}$ subunit and that the charge neutralization mutations in the mutant $\beta1$ subunit did not significantly affect the ability of $\beta1$ to increase the proportion of channels that recover rapidly from inactivation.

**DISCUSSION**

The Protein Component, but Not the Carbohydrate Component, of the Extracellular Domain Is Required for $\beta1$ Function—Deletion of the intracellular domain of the $\beta1$ subunit does not affect modulation of the brain type $IIA \alpha$ subunit, but deletion of segments of the extracellular domain does prevent Na$^+$ channel modulation by $\beta1$ subunits. Similarly, deletion analyses showed that the intracellular domain of human $\beta1$ is not required for modulation of the skeletal muscle $\alpha$ subunit by $\beta1$ subunits, whereas the extracellular domain is sensitive to deletion mutagenesis (15). In addition, the $\beta1$ extracellular domain, together with proximal residues of the transmembrane domain, was found to be sufficient for modulation of the skeletal muscle Na$^+$ channel in chimeric subunits formed with Na$^+$ channel $\beta2$ subunits (16). Taken together, these studies demonstrate that the extracellular domain is required for modulation of both brain and skeletal muscle $\alpha$ subunits.

The extracellular domain of the $\beta1$ subunit contains four potential sites for N-linked glycosylation, in good agreement with biochemical results (21). In the structurally related myelin P0 protein, the carbohydrate chain linked to the E-F loop is required for homophilic association and is thought to be located in a pocket of the Ig fold motif where it may serve to orient the molecule in relation to the membrane surface (24, 33). As in myelin P0, the putative glycosylation sites of $\beta1$ are localized to loops between the $\beta$ strands of the Ig fold, including two sites in the E-F loop. In contrast to myelin P0, our results show that the deletion of any of the four potential sites, as well as the simultaneous deletion of tandem E-F loop sites, does not significantly compromise the expression or function of $\beta1$. Therefore, we infer that carbohydrate-protein interactions are not significantly involved in either the association of the $\beta1$ subunit with the $\alpha$ subunit or the subsequent modulation of Na$^+$ channel gating by $\beta1$, as determined by the expression of Na$^+$ channels in *Xenopus* oocytes. Evidently, although the extracellular domain is very sensitive to alteration of protein structure by deletion mutagenesis, N-linked glycosylation of these specific sites is not required for function of the $\beta1$ subunit.

Core $\beta$ Sheets of the Ig Fold, but Not the Connecting Loops, Are Required for $\beta1$ Function—The results of the alanine-scanning mutagenesis of hydrophobic residues within the core $\beta$ strands support the hypothesis that the extracellular domain of $\beta1$ contains an essential Ig fold motif. All mutations that altered clusters of hydrophobic residues in the core $\beta$ strands prevented $\beta1$ function, as expected if they disrupted $\beta$ sheet interactions in the core of the Ig fold. If the hydrophobic core of the Ig fold was disrupted by the cluster mutations, crucial $\alpha/\beta1$ interaction sites may be lost because of the misfolding of the Ig fold scaffold, or the mutations may have conferred a decrease in the expression or stability of $\beta1$. In contrast, most mutations of the peripheral $\beta$ sheets or connecting loops within the Ig fold did not alter $\beta1$ function. Normal $\beta1$ function was also observed for $\beta1$ subunits carrying mutations in the juxtamembrane region, which is proposed to lie outside of the Ig fold. Therefore, our working hypothesis is that the core of the Ig fold is required to position specific molecular determinants appropriately for interaction and modulation of the Na$^+$ channel $\alpha$ subunit.

Our studies of chimeric proteins in which putative loop segments connecting the $\beta$ strands of $\beta1$ were replaced with the corresponding myelin P0 sequence lend additional support to the Ig-fold hypothesis. In contrast to our deletion and alanine-scanning analyses, it is noteworthy that substitutions of up to seven amino acids are tolerated in the connecting loops in the Ig fold of the $\beta1$ extracellular domain. These results are consistent with the Ig fold structure because these loops are not required for the structural integrity of the Ig fold. Mutations in loop regions not directly involved in interactions with the $\alpha$ subunit would be expected to have little or no effect on $\beta1$ function. From this analysis, we can exclude many of the amino acid residues in the connecting loops of the Ig fold from participating directly in interactions with the $\alpha$ subunit.

Acidic Residues in the $A/A'$ Strand Are Required for Full $\beta1$ Function—A region on the surface of the Ig fold was identified which is essential for optimum modulation of gating of the $\alpha$ subunit. Neutralization of negative charges (E4QD6N8E8Q) in the proposed $A/A'$ strand of the $\beta1$ Ig fold domain yielded Na$^+$ channels with very interesting electrophysiological characteristics. Two types of data argue that this mutant $\beta1$ subunit assembles properly and binds effectively to the $\alpha_{IIA}$ subunit. First, it competes for expression with the wild-type $\beta1$ subunit. Second, in experiments studying recovery from inactivation, no slow phase of recovery was observed, indicating that there were no free $\alpha$ subunits present. Despite being bound to each $\alpha$ subunit, this mutant $\beta1$ subunit consistently conferred rates of Na$^+$ channel inactivation which were intermediate between those seen for the $\alpha_{IIA}$ subunit coexpressed with the wild-type $\beta1$ subunit and those of channels consisting of only the $\alpha_{IIA}$ subunit (Fig. 7, panels A and B). Further analysis revealed that the fraction of channels that gate in the fast mode was significantly reduced in channels containing the $\beta1$(E4QD6N8E8Q) mutation (Fig. 7C). Taken together, the data suggest that the neutralization of negative charges in the $A/A'$ strand reduces the change in gating mode effected by the $\beta1$ subunit without preventing the association of $\beta1$ with the $\alpha$ subunit. Interestingly, because the $A$ and G strands interact with each other in Ig V-like motifs (23, 25), the lack of effect of mutations in the G strand of mutant AS-8 (Fig. 4C, trace i) supports the conclusion that the effects of mutations of charged amino acid residues in the $A/A'$ strand result from altered interactions with the $\alpha_{IIA}$ subunit rather than altered interactions with the G strand and the core of the Ig fold. The edge of the $\beta1$ Ig fold domain that carries these negatively charged residues may interact with the $\alpha$ subunit in a manner that affects its rate of inactivation and gating mode.

Comparison with Cell Adhesion Molecules—Members of the Ig superfamily are typically involved in cell recognition and cell adhesion. The inclusion of the Na$^+$ channel auxiliary subunits $\beta1$ and $\beta2$ in this superfamily suggests that these subunits may have additional functions beyond modulation of channel expression and gating properties. This hypothesis is supported by the observation that the primary sequence of $\beta2$ has a region with significant homology to the cell adhesion molecule contactin (5). It is interesting that the $A/A'$ strand of the $\beta1$ Ig fold, which we found to be important for interactions with the $\alpha$ subunit, is not among the Ig fold regions that are most often implicated in ligand binding or cell adhesion. For example, in antibodies and T-cell receptors, two variable domains dimerize to form an antigen binding site composed of residues within the
B-C, C'-C", and F-G loops, known as complementarity determining regions. Head-to-head homophilic adhesion of Ig V-like domains, such as those from CD2, typically involves the broad A'GFCC'C" face, and binding to integrins occurs primarily at the GFC face of cell adhesion molecules (23). In myelin P0, hydrogen bonds between residues in the C9 strand of partner molecules are believed to be the most important determinants of homophilic adhesion (25). The localization of α-β1 modulation determinants to a discrete edge of the Ig fold leaves the faces of the Ig fold and the loops analogous to the complementarity determining regions free for interactions with proteins from the extracellular matrix or opposing cell membranes.

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Molecular Determinants of Na\(^+\) Channel Function in the Extracellular Domain of the \(\beta_1\) Subunit
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