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Definition of Epitopes for Monoclonal Antibodies Developed Against Purified Sodium Channel Protein: Implications for Channel Structure

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Abstract. To test sodium channel structural models, we defined the epitopes for nineteen independently cloned monoclonal antibodies previously generated against purified, detergent-solubilized, adult rat skeletal muscle sodium channel protein using channel proteolysis, synthetic peptides, and fusion proteins. All identified epitopes were continuous and unique to the skeletal muscle subtype α-subunit. Of the nineteen independent clones, seventeen had epitopes located either in the origin of the amino-terminus or in the interdomain 2–3 region while only two antibodies had epitopes located in the midportion of the interdomain 1–2 region. No immunogenic regions were identified on the α-subunit's extracellular regions, interdomain 3–4 segment, or carboxyl-terminus or on channel β-subunits. While immune tolerance may explain the lack of immunogenicity of extracellular regions, the lack of immunogenicity of most of the channel's cytoplasmic mass may be due to segment inaccessibility from organization of these regions as globular domains, to insertion of parts of these regions into the membrane phase, or to interaction with other protein elements. The definition of monoclonal antibody epitopes allows us to reinterpret previously reported monoclonal antibody competition studies, providing independent support for our model of sodium channel cytoplasmic domain structure. In addition, these data suggest additional testable hypotheses concerning the interactions of the sodium channel amino- and carboxyl-termini with each other as well as with other protein elements.

Key words: Sodium channel — Antibody epitope — Protein structure — Proteolysis —ELISA — Fusion protein

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Introduction

Antibodies have been useful as both structural probes and immunocytochemical markers of the excitable membrane sodium channel. In early studies, antibodies developed against purified rat skeletal muscle sodium channel protein were used to map antibody binding sites (Casadei & Barchi, 1987) and to investigate sodium channel developmental (Haimovich, Tanaka & Barchi, 1986; Schotland, Fieles & Barchi, 1991) and tissue-specific expression (Haimovich et al., 1984). More recent studies have used antibodies with defined epitopes to probe channel structure at a more detailed level (Gordon et al., 1987*a;* Gordon et al., 1988; Thomsen & Catterall, 1989; Zwerling, Cohen & Barchi, 1991; Beck et al., 1993; Sun et al., 1995*a*) and to examine the expression of different sodium channel subtypes during development and following injury in different tissues (Elmer et al., 1990; Devor, Govrinlippmann & Angelides, 1993; Lupa et al., 1993; England et al., 1994; Vabnick et al., 1996). The ability to define the epitopes for monoclonal antibodies used in the earlier studies offers us the opportunity to test sodium channel structural models developed in the later studies and is the focus of this work.

In the mid-1980s, three separate fusions were used to generate a large panel of independently cloned monoclonal antibodies against purified, detergent-solubilized, adult rat skeletal muscle sodium channel (rSkM1) protein (Casadei et al., 1984). Selected members of this panel were used in a variety of biochemical, immunologic, and immunocytochemical studies (*see* Cohen & Barchi, 1993 for review). In one study, a subset of these antibodies was metabolically labeled and used in a solid-phase competition assay with unlabeled monoclonal antibodies to develop a map of antibody binding sites (Casadei & Barchi, 1987). This map identified three

families of antibodies whose epitopes were thought to be in close proximity on channel cytoplasmic segments and a fourth noninteracting family whose epitopes were thought to reside extracellularly. Immunocytochemical studies with two monoclonal antibodies produced three distinct patterns of immunolabeling in fast- and slow-twitch skeletal muscle surface and t-tubular membranes (Haimovich et al., 1987). These results were originally thought to reflect either the presence of different sodium channel subtypes in these tissues or differing epitope accessibility in these different membrane environments.

We later used sequence-specific, site-directed polyclonal antibodies to probe channel structure using exogenous proteolysis (Zwerling et al., 1991). Four protease-resistant limit fragments corresponding to the four homologous membrane embedded domains were identified and a hierarchy of structure for channel cytoplasmic segments was suggested. In this model, the carboxyl-terminus was modeled to be present in an extended conformation while the amino-terminus was modeled to be present in a compact, folded structure. More recently, a solution phase antibody competition assay allowed a more detailed model of the three dimensional organization of channel cytoplasmic segments to be proposed in which domains 1 and 4 were adjacent and a specific interaction between the sodium channel's amino- and carboxyl-termini was identified (Sun et al., 1995*a*). In that study, the segment involved in interacting with the carboxyl-terminus was found to reside within residues 1–30 of the amino-terminus.

In this paper, we define the epitopes for these remaining monoclonal antibodies using sodium channel proteolytic fragments, fusion proteins, and synthetic peptides. This information allows us to convert the previous map of antibody binding sites into a map of the relative organization of specific portions of the sodium channel's structure. In addition, the definition of antibody epitopes provides insights into the different patterns of immunocytochemical labeling obtained with these antibodies in adult skeletal muscle. The results reported here provide independent evidence that supports our model of the organization of channel cytoplasmic segments and strongly suggest that additional protein elements interact with sodium channel cytoplasmic segments.

Materials and Methods

Materials

All chemicals, enzymes, and chromatographic media were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified. Immulon 2 flat bottom microtitre plates were obtained from Dynatech Laboratories (Chantilly, VA). Rabbit anti-mouse IgG was obtained from Organon Teknika (Durham, NC). Immunologic grade ^{125}I-Protein A was obtained from Amersham (Irvine, CA).

Monoclonal Antibodies

Monoclonal antibodies used in this study were generated against purified adult rat skeletal muscle sodium channel protein (rSkM1) (Casadei et al., 1984) and have been extensively characterized (Casadei & Barchi, 1987; Casadei, Gordon & Barchi, 1986; Casadei et al., 1984; Haimovich et al., 1984; Haimovich et al., 1987; Haimovich et al., 1986). Protein used for immunization was purified from detergent-solubilized adult rat skeletal muscle membranes using sequential ion exchange chromatography on a Guanidinium-Sepharose column followed by lectin affinity chromatography on a Wheat germ-Sepharose column (Barchi, Cohen & Murphy, 1980). Nineteen of the original 23 independent monoclonal antibody clones were still available in sufficient titer to be used for these experiments. All antibodies were previously demonstrated to comigrate with ^{3}H-saxitoxin binding activity in sucrose gradient sedimentation studies, to immunoprecipitate ^{3}H-saxitoxin binding activity from crude solubilized rat skeletal muscle sarcolemmal membranes, and to identify either surface or surface and t-tubular membranes using immunofluorescence microscopy of fresh frozen adult rat skeletal muscle (Casadei & Barchi, 1987; Casadei et al., 1986; Casadei et al., 1984; Haimovich et al., 1984; Haimovich et al., 1987; Haimovich et al., 1986). All monoclonal antibodies specifically bind to the 276 kDa rSkM1 α-subunit on immunoblots; none bind to the channel's β-subunit (Kraner, Yang & Barchi, 1989; Kraner, Tanaka & Barchi, 1985). Epitopes for four of these monoclonal antibodies were previously determined (Cohen & Barchi, 1992; Sun, Cohen & Barchi, 1995*b*).

Peptide Synthesis

Peptides were synthesized using t-Boc chemistry on an Applied Biosystems 430A peptide synthesizer by the Protein Chemistry Laboratory of the University of Pennsylvania School of Medicine and cleaved with hydrogen fluoride. All peptides were greater than 95% pure as assessed by reverse phase HPLC using a C18 column on an Applied Biosystems 130A microbore HPLC system. Amino acid composition was confirmed by direct analysis. Nested oligopeptides comprising portions of the 1-30 peptide (encoding residues 1-30 of the rSkM1 sequence) were obtained by removing resin containing nascent peptide at different stages during synthesis of the 1-30 peptide, giving rise to the following peptides which are named based on the residues they contain: 24-30, 19-30, 13-30, 7-30, and 1-30. A peptide encoding residues 1-12 was also synthesized.

Construction of Fusion Proteins

Fusion proteins comprising the full length rSkM1 interdomain 2–3 and carboxyl-terminus and different length fragments of the rSkM1 interdomain 2–3 region were expressed and purified using the maltose-binding protein (MBP) fusion and purification system (New England Biolabs, Beverly, MA), as previously described (Sun et al., 1995*b*). An identical approach was used to generate both full-length and fragments of the interdomain 1–2 region. Briefly, PCR primers were designed based on the length and location of the interdomain 1–2 fragments. Sense primers (5′ end) all contained an *Eco*RI site, while antisense primers (3′ end) all contained an *Xba1* site. PCR reactions were performed using 1 μg of pCMV/rSkM1 (containing the full length rSkM1 cDNA) and *Taq* DNA polymerase (5 units, EC 2.7.7.7, Boehringer Mannheim). PCR products underwent electrophoresis on agarose gels, were purified by Gene Clean (Bio101, LaJolla, CA), and were then double digested with the enzymes *Eco*RI and *Xba1*. All purified fragments were ligated with the *Eco*RI- and *Xba1*-digested

pMal-cRI vector. Constructs were then transformed into *Escherichia coli* (TB1 strain), amplified and sequenced. Fusion proteins containing the following rSkM1 interdomain 1–2 residues were produced: 445-566 (full length), 445-545, 445-520, 445-495, and 445-470.

An epitope-tagged protein containing the rSkM1 amino-terminus was similarly constructed and expressed using the IBI FLAG expression system (Eastman Kodak, New Haven, CT). Briefly, the FLAG marker octapeptide was joined to the rSkM1 amino-terminus (residues 1-127) by inserting the amino-terminal coding sequence upstream from the FLAG gene between the *Nde1* (5′ end) and *Bgl2* (3′ end) of the pFLAG.CTC vector.

Sodium Channel Purification and Proteolysis

Sodium channel protein was purified and proteolyzed as described in a previous publication (Zwerling et al., 1991). Briefly, skeletal muscle membranes were solubilized using 1% NP-40 and successively purified using ion exchange chromatography on diethyl amino-ethyl (DEAE) Sephadex and lectin affinity chromatography on Wheat Germ Agglutinin (WGA)-Sepharose. Purified sodium channel protein in detergent micelles was proteolyzed with 0.5 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin type XIII (EC 3.4.21.4, Sigma) at room temperature; aliquots of the reaction mixture were removed at the time points indicated on the individual gels in Fig. 1. The reaction was quenched by adding sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heating the sample to 98°C for 5 min. Zero time point samples contained no protease. Samples were treated with iodoacetamide and stored at −70°C until subjected to SDS-PAGE.

Immunoblotting

Protein samples underwent SDS-PAGE and immunoblotting as previously described (Zwerling et al., 1991). Blots were incubated in either a 1:10 or a 1:100 dilution of mouse monoclonal antibody supernatant in 2% Bovine serum albumen in phosphate buffered saline (BSA-PBS), a 1:1,000 dilution of mouse monoclonal anti-FLAG IgG (IBI, Eastman Kodak, New Haven, CT), or a 1:10,000 dilution of polyclonal rabbit anti-MBP serum (New England Biolabs, Beverly, MA) in 2% BSA-PBS for 2 hr at room temperature. For polyclonal antibodies, blots were then washed five times with PBS containing 0.25% Tween-20 and incubated with 1.25 μCi/ml ^{125}I-Protein A (ICN, Irvine, CA). For blots in which the primary antibody was a mouse monoclonal antibody, blots were first incubated with affinity-purified rabbit anti-mouse F(ab_2) (Zymed, San Francisco, CA) at a 1:1,000 dilution in 2% BSA-PBS for 2 hr at room temperature. Bound radioactivity was visualized by autoradiography with Kodak XAR-5 film using a Cronex intensifying screen (DuPont, Wilmington, DE).

Epitope Localization Using ELISA

The enzyme-linked immunosorbent assay (ELISA) was used to assess binding of peptides by monoclonal antibodies. Peptides (1 uM in PBS) were added to each well of a microtiter plate and incubated at room temperature overnight. Unbound sites were then blocked with 1% BSA/PBS for 1 hr. Antibodies diluted in 1% BSA/PBS were incubated for 1 hr at room temperature. Following successive washes of 0.25% Tween 20/PBS, horse-radish-peroxidase conjugated anti-mouse antibody diluted 1:10,000 was added and allowed to incubate for 1 hr at room temperature. After washing, o-phenylenediamine dihydrochloride (OPD) substrate solution was added. Following a 30-min incubation, the reaction was quenched with 3M-HCl and the reaction mixture quantitated at an absorbance of 490 nm. Competition assays were performed by preincubating 1:10 dilutions of monoclonal antibody for 1 hr at room temperature with the indicated concentrations of oligopeptides in 1% BSA/PBS prior to use in the ELISA. All ELISAs were performed in triplicate and each experiment was repeated at least three times.

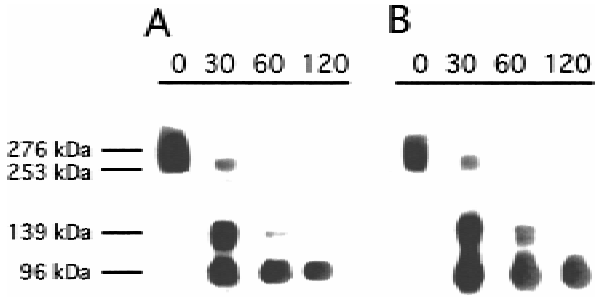

Fig. 1. Initial epitope localization using channel proteolysis: Purified adult rat skeletal muscle sodium channel protein in detergent micelles was subjected to proteolysis using trypsin. Aliquots of the reaction mixture were removed at the indicated time points, the reaction quenched with sample buffer and heating to 98°C for 5 min, and the samples subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting. The blot shows the specific temporal sequence of fragment appearance. Although all proteolytic fragments are present in each lane, only those containing the epitope of interest are visualized. (*Panel A*) Polyclonal antibody I-31, generated against residues 31-47 in the rSkM1 sequence, identifies the intact 276 kDa α-subunit, the slightly proteolyzed 253 kDa α-subunit (30 min), and the appearance of discrete bands at 139 kDa and 96 kDa (30, 60, 120 min). (*Panel B*) Unknown monoclonal antibody F/E4 identifies the identical fragments in the same temporal sequence. Our previous proteolysis study (Zwerling et al., 1991) identified the 96 kDa limit fragment as containing residues comprising the amino-terminus and the first homologous Domain, thus localizing the F/E4 epitope to this channel fragment.

Results

Initial epitope localization was achieved by assessing monoclonal antibody binding to sodium channel proteolytic fragments on immunoblots. Proteolysis occurs in discrete, reproducible steps; while all channel fragments are present in each sample, each antibody only identifies fragments containing that specific antibody's epitope. Because of differences in the size and number of fragments identified by each of our panel of sequence-specific polyclonal antibodies (Zwerling et al., 1991), the size and pattern of fragments identified with each monoclonal antibody allow us to discern its epitope's regional location. All antibodies identified fragments associated with either (i) the amino-terminus and first homologous Domain; (ii) the interdomain 1–2 and second homologous Domain; or (iii) the interdomain 2–3 and third homologous Domain (Fig. 1). These initial epitope localizations were confirmed by either stripping blots and reprobing with site-specific polyclonal antibodies (Zwerling et al., 1991) or by probing identical blots generated

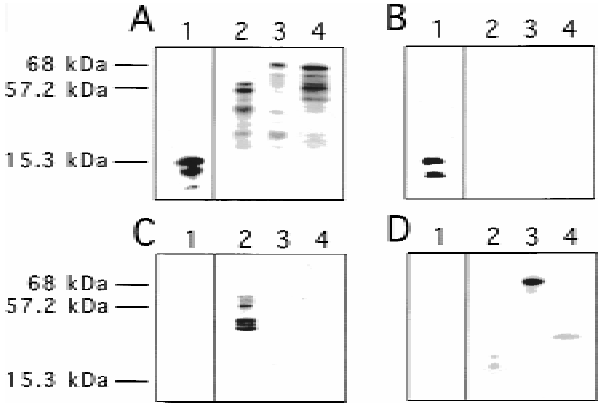

Fig. 2. Epitope localization using fusion proteins: Fusion proteins containing the rSkM1 amino-terminus tagged with the FLAG epitope at its carboxyl-terminus (Lane 1) or the interdomain 1-2 (Lane 2), interdomain 2-3 (Lane 3), or carboxyl-terminus (Lane 4) linked to maltose-binding protein at their carboxyl-termini were electrophoresed and immunoblotted. (*Panel A*) anti-FLAG monoclonal antibody (Lane 1) or anti-MBP polyclonal antibody (Lanes 2–4) were used to identify expressed fusion proteins. (*Panel B, C,* and *D*) Monoclonal antibodies F/E4, G/B10, and D/F9, respectively, were incubated with blots containing each of the fusion proteins. Each antibody bound specifically only to a single fusion protein, identifying the epitope locus for F/E4 in the amino-terminus, G/B10 in the interdomain 1-2 region, and F/E4 in the interdomain 2-3 region. No monoclonal antibody bound to the fusion protein containing the carboxyl-terminus of the rSkM1 sodium channel.

in parallel with both monoclonal (epitope unknown) and polyclonal (epitope known) antibodies.

We hypothesized that, due to their size and charge, the interdomain or terminal segments were more likely than parts of each homologous domain to contain antibody epitopes. Thus, we developed and tested fusion proteins comprising four of the five channel cytoplasmic segments for immunoreactivity with each of the monoclonal antibodies. As expected, each antibody bound to only one of these fusion proteins: eight monoclonal antibodies reacted specifically with the FLAG amino-terminal fusion protein, two with the MBP interdomain 1-2 fusion protein, and nine with the MBP interdomain 2-3 fusion protein (Fig. 2 and Table). None of the monoclonal antibodies reacted with the MBP carboxyl-terminal fusion protein. Anti-peptide sequence-specific polyclonal antibodies (Zwerling et al., 1991) and either anti-FLAG or anti-MBP antibodies served as positive controls for these studies.

We used previously synthesized peptides corresponding to portions of almost the entire rSkM1 amino-terminus (i.e., peptides 1-30, 31-46, 71-101, and 97-126) to further localize epitopes for monoclonal antibodies which bound to the amino-terminal fusion protein. All antibodies bound specifically only to the peptide encoding rSkM1 residues 1-30 (Fig. 3*a*), identifying this as the epitope-containing segment. Further localization of

Table. Regional location with specific epitope residues

	Epitope (residues)
Amino-terminal epitopes	
A/B2, F/E4, F/G6, G/G8, G/G9, I/E3	1-6
D/E6	13-18
L/D3	19-24
Interdomain 1-2 epitopes (mid-region)	
D/C12, G/B10	495-520
Interdomain 2-3 epitopes (mid-region)	
D/F9, F/C11	865-875
Interdomain 2-3 epitopes (end-region)	
A/C12, B/B1, B/D6, B/H4, E/F6, M/H1, P/D4	965-975

these antibody epitopes was achieved using an ELISA assay in which a nested set of peptides corresponding to successively larger segments of the 1-30 sequence (i.e., 24-30, 19-30, 13-30, and 7-30) as well as a peptide encoding residues 1-12 were tested for immunoreactivity. Six of the monoclonal antibodies bound specifically only to the peptide encoding residues 1-12 (Fig. 3*b*), thus localizing the epitope for these antibodies to residues 1-6 (*see* Table). Antibody D/E6 bound only to peptides 13-30 and 7-30, localizing its epitope to residues 13-18; a competition assay in which the D/E6 antibody was first preabsorbed with each peptide before being assayed for immunoreactivity with peptide 1-30 was used to confirm this epitope localization (*see* Fig. 3*c* and Table). The epitope for antibody L/D3 was previously localized to residues 19-24 based on immunoreactivity with peptides 19-30, 13-30, and 7-30, but not 24-30 (Cohen & Barchi, 1992).

Further localization of the epitopes for the remaining antibodies was achieved using MBP fusion proteins encoding different length segments of either the interdomain 1-2 or interdomain 2-3 regions. One set of fusion proteins, comprising different fragments of the interdomain 2-3 region, was previously developed and used to identify the epitopes for monoclonal antibodies F/C11 and B/D6 as residues 865-875 and 965-975, respectively (Sun et al., 1995*b*). An identical approach was used to develop a second set of fusion proteins to localize epitopes for antibodies with epitopes in the interdomain 1-2 region. Panels of fusion proteins containing different length segments of both interdomain regions were then tested for immunoreactivity with monoclonal antibodies using immunoblot analysis. Anti-MBP monoclonal antibody and sequence-specific anti-peptide polyclonal antibodies served as positive controls. Using this approach, epitopes for monoclonal antibodies D/C12 and G/B10 were localized to residues 495-520 in the interdomain 1-2 region (Fig. 4), epitopes for monoclonal antibodies D/F9 and F/C11 were localized to residues 865-875 in the mid-portion of the interdomain 2-3 region, and epitopes for the remaining seven monoclonal antibodies were localized to residues 965-975 in the far portion of the interdomain 2-3 region (*see* Table).

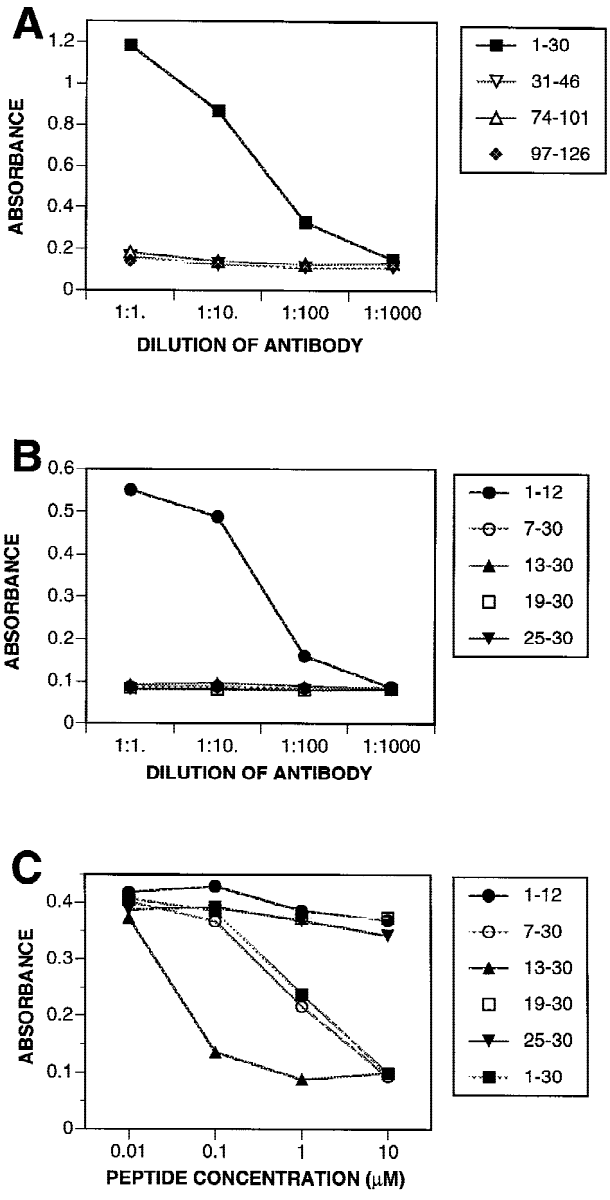

Fig. 3. Localization of monoclonal epitopes using synthetic peptides: (*Panel A*) Each of the monoclonal antibodies whose epitope was initially localized to the amino-terminus and Domain 1 was tested in an ELISA assay against synthetic peptides comprising most of the rSkM1 amino-terminus (peptides 1-30 (■), 31-46 (▼), 74-101 (△), and 97-126 (❖)). All monoclonal antibodies (F/E4 is shown) bound specifically only to the 1-30 peptide. (*Panel B*) Further localization was achieved using a nested set of peptides corresponding to successively larger portions of the 1-30 peptide (i.e., peptides 7-30 (○), 13-30 (▲), 19-30 (□), and 25-30 (▼)) and peptide 1-12 (●) in an ELISA. Most of the monoclonal antibodies (F/E4 is shown) bound only to the 1-12 peptide, identifying residues 1-6 as the antibody epitope. (*Panel C*) Competition assay in which monoclonal antibody D/E6 was first incubated with each of the nested set of peptides before being applied to wells containing the 1-30 peptide. This assay was used to confirm the localization of monoclonal antibody D/E6 to residues 13-18. The epitope for monoclonal antibody L/D3 was localized to residues 19-24 in a previous study using an identical approach (Cohen & Barchi, 1992).

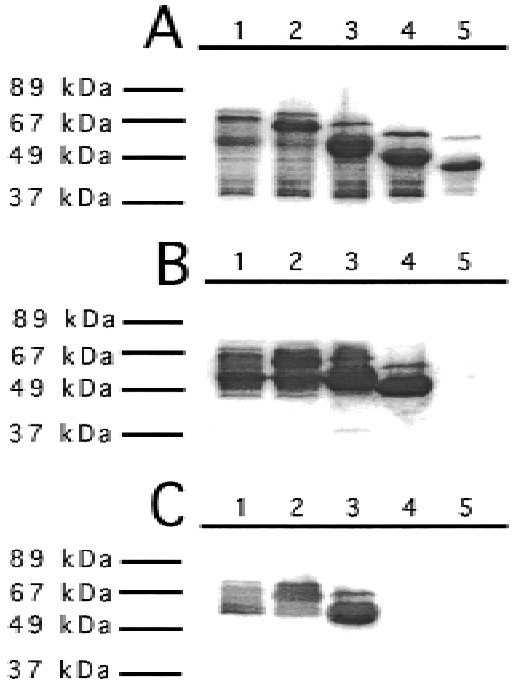

Fig. 4. Epitope localization in the interdomain 1-2 region: The epitopes for D/C12 and G/B10, monoclonal antibodies which bound to proteolytic limit fragments associated with the interdomain 1-2 and Domain 2 region, were further localized using a set of fusion proteins containing both full length and a nested set of fragments containing successively shorter segments of the interdomain 1-2 region. MBP fusion proteins underwent SDS-gel electrophoresis and immunoblot analysis. Lane 1 contains the fusion protein encoding the entire interdomain 1-2 region (residues 445-566). Lane 2 contains the fusion protein encoding residues 445-545. Lane 3 contains the fusion protein encoding residues 445-520. Lane 4 contains the fusion protein encoding residues 445-495. Lane 5 contains the fusion protein encoding residues 445-470. (*Panel A*) Blot probed with polyclonal rabbit anti-MBP serum. (*Panel B*) Blot probed with I-467, a polyclonal rabbit antibody generated against residues 467-486 in the rSkM1 sequence (Zwerling et al., 1991). (*Panel C*) Blot probed with monoclonal antibody G/B10. The anti-MBP antibody shows the pattern of MBP fusion proteins, ranging from an expected molecular mass of 57.2 kDa for the MBP—445-566 construct to 45.6 kDa for the MBP—445-470 construct; lower molecular weight bands represent proteolytic fragments of the full length fusion protein. As predicted, antibody I-467 identifies each of the fusion proteins except for the MBP—445-470 while monoclonal antibody G/B10 identifies the fusion protein containing residues 445-520 but not residues 445-495 or 445-470, thus identifying residues 495-520 as the segment containing the G/B10 epitope. Identical results were obtained with monoclonal antibody D/C12.

Discussion

Immunogenic Regions of the rSkM1 Sodium Channel Protein

All monoclonal antibody epitopes were continuous, unique to the skeletal muscle sodium channel subtype, and localized to four discrete regions in cytoplasmic seg-

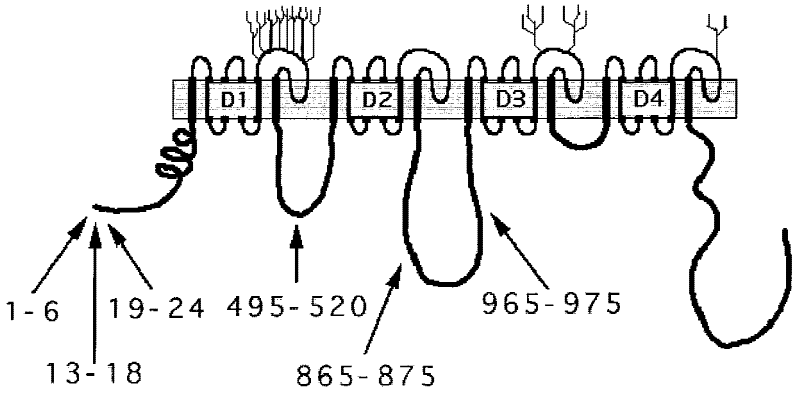

Fig. 5. Cartoon depicting location of monoclonal epitopes in the sodium channel: Two dimensional model of the sodium channel showing the four membrane embedded domains (labeled D1, D2, D3, and D4), predicted extracellular carbohydrate (tree structures on upper part of figure), and cytoplasmic segments (lower part of figure). The arrows denote the location of each group of monoclonal epitopes. The numbers associated with each arrow represent the specific amino acid residues of the adult rat skeletal muscle sodium channel which comprise each epitope.

ments of the adult rat skeletal muscle sodium channel protein (*see* Table and Fig. 5). Fifteen of the monoclonal antibody epitopes were localized in two regions: the origin of the amino-terminus or the far portion of the interdomain 2-3 region just prior to Domain 3. The four other epitopes were located in the mid-portions of the interdomain 1-2 and interdomain 2-3 cytoplasmic segments. This extremely limited number of antigenic sites is unexpected given that current models place ~70% of the channel's protein mass (1296 of 1840 residues) and all of the ~68 kDa of complex carbohydrate (Zwerling et al., 1991) in extramembranous locations.

While immune tolerance may provide an explanation for the lack of an immune response against extracellular protein or carbohydrate, this is certainly not the case for the substantial remaining cytoplasmic sequences. These sequences, which comprise ~42% of the rSkM1 protein, would be expected to be both physically available (in detergent micelles) and immunogenic based on our extensive experience in developing and using site-specific anti-peptide antibodies directed against these regions (Cohen, 1996; Cohen & Levitt, 1993; Sun et al., 1995*a*; Zwerling et al., 1991). However, the frequent occurrence of stretches of hydrophobic residues in these cytoplasmic segments suggests that, unlike current models which depict these regions as extending away from the membrane into the cytoplasm, these segments may form globular domains in the cytoplasm or, perhaps, fold back and enter the lipid bilayer. Thus, inaccessibility or steric hindrance may be a major reason for the lack of immunogenicity of these segments.

Inaccessibility or steric hindrance of the carboxyl-terminus may also be due to its interaction with other cytoplasmic proteins or peptides. We have already reported the specific interaction of the rSkM1 amino-terminus with the carboxyl-terminus (Sun et al., 1995*a*). A similar high affinity interaction between the rSkM1 interdomain 2-3 region and the carboxyl-terminus has also been observed (S.S. Kolibal, L.J. Chlumsky & S.A. Cohen, *manuscript in preparation*). In addition, two recent reports describe the interaction of syntrophin with certain sodium channels via their carboxyl-terminal PDZ domains (Gee et al., 1998; Schultz et al., 1998). Thus, interactions of the carboxyl-terminus with other parts of the sodium channel or with other proteins may also serve to reduce the immunogenicity of the region (*see* further discussion below).

Implications for the Interaction of rSkM1 Amino- and Carboxyl-termini

Early immunofluorescent studies with antibodies whose epitopes are now identified as residing in the amino-terminus produced three distinct patterns of labeling in immunocytochemistry of adult rat skeletal muscle (Haimovich et al., 1987). All amino-terminal antibodies specifically labeled the surface membranes of fast- and slow-twitch skeletal muscle cells. However, monoclonal antibodies with epitopes now known to encompass residues 1-6 also specifically labeled the t-tubular system of slow-twitch fibers while no antibodies labeled the t-tubular system of fast-twitch fibers.

One possible explanation of these findings is the presence of either other sodium channel subtypes or alternate splicing of the rSkM1 channel in skeletal muscle. While mRNA encoding rat brain sodium channel subtypes I and III was identified in adult rat skeletal muscle using PCR (Schaller et al., 1992), protein corresponding to these or other sodium channel subtypes have not been identified in this tissue (Gordon et al., 1987*b;* Mandel, 1992). In addition, alternate splicing has not been found for the rSkM1 isoform using either biochemical or molecular approaches (R.L. Barchi, *personal communication*).

Our recent identification of residues 1-30 in the rSkM1 sodium channel amino-terminus as the region which binds to the carboxyl-terminus (Sun et al., 1995), however, suggests alternate explanations for these immunocytochemical findings: (i) sodium channel protein in surface membranes either does not demonstrate binding of amino- and carboxyl-termini or does so in a man-

ner that does not produce steric hindrance to antibody binding to epitopes in the amino-terminal binding region; (ii) sodium channel protein in slow-twitch t-tubular membranes manifests the interaction of amino- and carboxyl-termini by interfering with binding of antibodies whose epitopes are in the middle of this region (i.e., antibody L/D3 with its epitope at residues 19-24) but not for antibodies with epitopes at the origin of the protein (i.e., A/B2 with its epitope at residues 1-6); and (iii) sodium channel protein in fast-twitch muscle t-tubular membranes demonstrates steric hindrance for antibody binding to all epitopes in the first 30 residues.

Therefore, the absence of antibody binding could be due to epitope unavailability due to alternate conformations of the amino- and carboxyl-terminal complex, some of which produce steric hindrance to antibody binding. Alternatively, the amino-terminus and/or the carboxyl-terminus may interact either with other channel cytoplasmic segments or with other cytoplasmic proteins in different membrane environments, as mentioned above. Recent findings in our laboratory demonstrating a specific interaction between the rSkM1 interdomain 2-3 region and the carboxyl-terminus (S.S. Kolibal, L.J. Chlumsky & S.A. Cohen, *manuscript in preparation*) suggest that the binding of other channel segments by the carboxyl-terminus may provide part of the explanation for these immunocytochemical findings. The recent report of the binding to syntrophin to sodium channel carboxyl-termini (Gee et al., 1998; Schultz et al., 1998) suggests that steric hindrance due to the physical proximity of other proteins is another possible explanation. This suggestion is supported by the observation of binding motifs in the sodium channel carboxyl-terminus for proteins involved in membrane protein trafficking, including di-leucine, Src homology 2, Src homology 3, and WW motifs (*see* Pawson & Scott, 1997 for review). This observation and the report of syntrophin binding to sodium channel terminal PDZ binding motifs provides support for the concept that the carboxyl-terminus may interact with one or more cytoplasmic proteins. Thus, steric hindrance due to specific patterns of protein binding in different membrane environments provides an attractive explanation for the immunocytochemistry findings.

Implications for the Organization of Sodium Channel Cytoplasmic Segments

Reexamination of the patterns of monoclonal antibody competition from the original monoclonal antibody paper (Casadei & Barchi, 1987) fits well with our groupings of antibodies based on epitope localization. The four groupings derived from the original competition data exactly match the four families of monoclonal antibodies identified by epitope location. In addition, three patterns of monoclonal-monoclonal antibody competition (absent, partial, and complete) were observed in the original studies (Casadei & Barchi, 1987) and are explained by our definition of antibody epitopes. Antibodies demonstrating complete competition exactly match the groupings of antibodies with identical epitopes as would be expected when two antibodies attempt to bind to the same epitope. Antibodies demonstrating partial competition bind to distinct epitopes; however, either the spheres of occupancy of the antibodies partially overlap or the native protein segment between the two epitopes is constrained in structure, thus orienting the antibodies in different directions. For example, although the epitopes for antibodies A/B2 and L/D3 are located only 12 residues apart, the previous observation of only partial competition despite increasing antibody titer is consistent with our model of this segment as a rigid structure: the antibodies demonstrate only partial inhibition because they are constrained to face in different directions.

No competition between metabolically labeled antibodies was observed for antibodies with epitopes in the two separate epitope regions in the interdomain 2-3 segment (Casadei & Barchi, 1987) even though they are only ~100 residues apart. Given that proteolysis studies indicate that the interdomain 2-3 is the most accessible of the interdomain regions to exogenous proteolysis, these results suggest that either sufficient distance between the two epitope or some defined structure between these two antigenic segments prevents the interaction of antibodies bound to this segment.

Finally, epitopes for two monoclonal antibodies mapped to the mid-portion of the relatively large (~125 residue) interdomain 1-2 region. The original monoclonal antibody competition studies found that these two antibodies did not exhibit competition with any other group of monoclonal antibodies (Casadei & Barchi, 1987). Since this segment was readily cleaved by and, thus, is accessible to exogenous proteases (Zwerling et al., 1991), these data are consistent with a model in which the interdomain 1-2 segment extends into the cytoplasm away from other cytoplasmic segments.

Thus, these data both support and extend selected features of our model of sodium channel cytoplasmic segments (Sun et al., 1995*a*). This model proposes that: (i) the amino-terminus assumes a rigid structure having a fixed orientation to other intracellular segments; (ii) the interdomain 2-3 region is centrally located, extends into the cytoplasm, and has an intermediate degree of flexibility; (iii) the amino- and carboxyl-termini interact; and (iv) domains 1 and 4 are adjacent. The present study is consistent with each of these predictions, especially with regard to the organization of the amino-terminus as a rigid structure with fixed orientation to other intracellular segments. The present study also suggests that: (i) unlike current models that depict cytoplasmic regions as extending away from the membrane into the cytoplasm,

certain portions of these segments (especially those containing stretches of hydrophobic residues) may form globular domains in the cytoplasm or, perhaps, fold back and enter the lipid bilayer; (ii) there is some defined structure between the two epitope segments in the interdomain 2-3 region that prevents the interaction of antibodies bound to this segment; and (iii) the interdomain 1-2 segment extends into the cytoplasm away from other cytoplasmic segments.

Circular dichroism and intrinsic fluorescent studies of the rSkM1 cytoplasmic segments expressed as soluble fusion proteins also suggest that these segments, in the absence of adjacent membrane domains, are organized into specific secondary structures and are able to mimic the interaction of the amino- and carboxyl-termini as originally observed in the intact channel protein (Zhang et al., *submitted*). Until crystallization of large membrane proteins becomes possible, crystallization of these cytoplasmic segments devoid of the membrane domains may help to provide support for our structural predictions.

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