Probing Sodium Channel Cytoplasmic Domain Structure

EVIDENCE FOR THE INTERACTION OF THE rSkM1 AMINO AND CARBOXYL TERMINI*

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Epitopes for monoclonal antibodies directed against the purified adult rat skeletal muscle sodium channel (rSkM1) were localized using channel proteolysis and fusion proteins. The interactions between these and other monoclonal antibodies with site-specific polyclonal antibodies were used to investigate the spatial relationships among rSkM1 cytoplasmic segments. Competition between antibodies for binding was performed using a solution-phase assay in which solubilized channel protein retains many of the biophysical characteristics of the rSkM1 protein in vivo. Our results support a model in which: 1) the amino terminus assumes a rigid structure having a fixed orientation with respect to other intracellular segments; 2) the interdomain 2–3 region is centrally located on the cytoplasmic surface of the channel, extends farther into the cytoplasm, and has an intermediate degree of flexibility; 3) the beginning of the amino terminus and end of the carboxyl terminus specifically interact with each other; and 4) domains 1 and 4 are adjacent. The sequences responsible for the interaction of the amino and carboxyl termini were identified by demonstrating the specific binding of a synthetic peptide encompassing the first 30 residues of the rSkM1 amino terminus to a fusion protein containing the rSkM1 carboxyl terminus.

EXPERIMENTAL PROCEDURES

Materials—Materials for the preparation of oligopeptides and antibodies, the isolation of crude membranes, and for the purification of membrane proteins were obtained from sources previously identified (8). DEAE-Sephadex (A25-120), wheat germ agglutinin-agarose, protease inhibitors, and one set of prestained molecular mass standards (26.2–180 kDa) were obtained from Sigma. Another set of prestained molecular mass standards (15–110 kDa) was obtained from Bio-Rad. [125I]-protein A and [125I]-labeled goat anti-mouse IgG were from ICN Radiochemicals (Irvine, CA). The pMal fusion protein and purification system was obtained from New England Biolabs.

Preparation of Antisera—Monoclonal antibodies were previously generated against purified sodium channel protein. Oligopeptides were synthesized and polyclonal antibodies generated against these synthetic oligopeptides using methods detailed in earlier publications (8–10). All oligopeptides correspond to regions of the rat skeletal muscle sodium channel protein sequence (Fig. 1 and Table I). The carboxyl-terminal cysteine residue which terminates each peptide is not part of the naturally occurring sequence and was added to assist in coupling peptide to carrier protein prior to rabbit immunization.

Sodium Channel Protein Purification—Preparation of muscle surface membranes containing unproteolyzed sodium channels was identical to that described previously (8). Sodium channel protein was solubilized using Nonidet P-40 and then purified using sequential ion exchange and lectin affinity chromatography (8). Purified solubilized sodium channel protein was kept at 4 °C and used within 2 weeks.

Antibody Binding Assay—Purified rSkM1 protein (5 pmol) was adsorbed to 25 μl of wheat germ lectin-Sepharose 4B by gentle agitation for 1 h at room temperature. The resin was pelleted and washed with

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Fig. 1. Antibody location and relative position. Two-dimensional model of the sodium channel with the relative positions of each antibody used in this study. Monoclonal antibodies are indicated by boxes, polyclonal antibodies by ovals. The amino terminus is present on the left side while the carboxyl terminus is present on the right side of the figure. D1 refers to domain 1, D2 to domain 2, etc. Refer to Table I for details of the residues comprising each antibody epitope.

Monoclonal Epitope Localization—Having localized the epitopes for representative members of one group of monoclonal antibodies (A/B2 and L/D3) to the amino terminus of rSkM1 in a previous study (12), our initial goal was to identify the epitopes for two representative members (F/C11 and B/D6) of a second group of monoclonal antibodies. Regional localization was obtained by comparing the pattern of proteolyzed rSkM1 sodium channel fragments that bound these antibodies to the patterns already described for sodium channel fragments visualized by antibodies directed against known epitopes distributed throughout the channel primary structure (8). The pattern of fragments as well as the limit peptide identified by antibodies F/C11 and B/D6 indicates that the epitopes for these two monoclonal antibodies lie within a region encompassing interdomain 2-3 and domain 3 (Fig. 2).

These epitopes were further localized to the interdomain 2-3 region on the basis of antibody binding to a fusion protein that contained residues 794-1014 in the rSkM1 sequence. Both antibodies reacted specifically with this fusion protein both on Western blots and in radioimmunoassay. We refined this localization using fusion proteins containing successively smaller fragments of the interdomain 2-3 region. These fusion proteins were constructed using either naturally occurring restriction sites in the coding sequence or synthetic primers in conjunction with the polymerase chain reaction (see Sun et al. (14) for details). Our binding data (Fig. 3) restricts the epitope for F/C11 to residues 865–875 (mid-portion of interdomain 2-3) and the epitope for B/D6 to residues 965–975 (carboxyl-terminal half of interdomain 2-3) just prior to domain 3) in the rSkM1 sequence.

Antibody Competition Studies—To investigate the spatial relationships of sodium channel intracellular segments, four representative monoclonal antibodies were assayed for binding competition with a panel of polyclonal antisera for which defined epitopes are already known (Table I). Measurements were made with solubilized purified rSKM1 sodium channel protein in a mixed micellar form that retains native channel structure, as indicated by toxin binding activity (15), sensitivity to proteolytic enzymes (8), and capacity for functional reconstitution (16, 17). Channel protein was immobilized to wheat germ-Sepharose beads. We first measured the kinetics of

Table I

| Name | Type | Location     | Sequence Epitope |
|------|------|--------------|-----------------|
| A/B2 | M    | N terminus   | 1-6             | MASSEL          |
| L/D3 | M    | N terminus   | 19-24           | PFTPES          |
| I-31 | P    | N terminus   | 31-46           | AVEEELORLKENQMEIC |
| I-467| P    | Interdomain 1-2 | 467-484  | EKYKHHQEOELEKAAQAQC |
| F/C11| M    | Interdomain 2-3 | 865-875  | STPEDEKKEPP |
| B-30 | P    | Interdomain 2-3 | 921-935  | SEDSEMPEETEETDC |
| B/D6 | M    | Interdomain 2-3 | 965-975  | PPEEPDPEEAE |
| R-12 | P    | Interdomain 3-4 | 1312-1323 | YNANMKKLSGSKLC |
| B-23 | P    | C terminus   | 1598-1611     | TESSESEPLSEDFEC |
| I-1771| P  | C terminus   | 1771-1791     | KMYGHEKEKGDGVQSQGEKAC |

1 The abbreviations used are: PBS, phosphate-buffered saline; MBP, maltose-binding protein.
276 kDa
63-69 kDa
31-35 kDa

A B

A B

A B

A B

B-30
F/C11
B/D6
B-23

Fig. 2. Initial localization of F/C11 and B/D6 epitopes. Western blot depicting nonproteolyzed (A) and limit digests (B) of rSkM1 protein developed with the indicated antibody. Limit digestes were obtained by treating 5 pmol of purified sodium channel protein for 120 min with 1-chloro-3-tosylamido-7-amino-2-hepanone-α-chymotrypsin (0.5 µg/ml) at room temperature (B). All antibodies identify the 276-kDa subunit in the nonproteolyzed samples. The pattern of the limit digest indicates that the epitopes for the two unknown monoclonal antibodies (F/C11 and B/D6) are located on the same limit fragments as the epitope for B-30 (i.e. interdomain 2–3 and domain 3).

38 kDa
36 kDa

(AA: 941)

LH

SKM Interdomain 2-3
d

IMMUNOREACTIVITY

F/C11

B/D6

(AA:1017)

MMP

+ +

AA: 794-1017

MMP

+ +

AA: 794-960

MMP

+ +

AA: 794-942

MMP

+ +

AA: 794-920

MMP

+ +

AA: 794-910

MMP

+ +

AA: 794-880

MMP

+ +

AA: 794-875

MMP

+ +

AA: 794-865

MMP

+ +

AA: 794-856

MMP

+ +

AA: 931-975

MMP

- +

AA: 931-965

MMP

- +

Fig. 3. Localization of monoclonal epitopes using fusion proteins. Schematic diagram of the panel of fusion proteins used to localize the epitopes for F/C11 and B/D6. AA refers to amino acid residues in the rSkM1 protein sequence. A "+" indicates that the respective antibody identified the indicated fusion protein in radioimmunoassays and on Western blots, while a "−" indicates that there was no reactivity of the indicated fusion protein with the respective antibody.

We tested this hypothesis by examining the interactions between fragments of various channel cytoplasmic segments in a solution phase binding assay. Fusion proteins containing the bacterial MBP joined at its carboxy-terminal end to various rSkM1 or rSkM2 cytoplasmic segments were prepared. Fusion proteins containing the rSkM1 or rSkM2 carboxyl termini, the rSkM1 interdomain 2–3 region, or MBP alone were immobilized on amyllose resin and incubated with a synthetic peptide corresponding to residues 1–30 of the rSkM1 amino acid sequence (1–30 peptide), the region containing the A/B2 and L/D3 epitopes. Bound peptide was identified by subsequent incubation of the peptide-fusion protein-resin complex with either A/B2 or L/D3 followed by 125I-labeled goat anti-mouse IgG.

We found no specific binding of the 1–30 peptide to fusion proteins containing the rSkM2 carboxyl terminus, the rSkM1 interdomain 2–3, or the MBP alone. However, the peptide bound specifically and with high affinity to the rSkM1 sodium channel carboxyl-terminal fusion protein (Fig. 5).

DISCUSSION

Based on previous estimates (18), the absence of competition between two antibodies for binding suggests that their epitopes are located more than 3.5 nm apart or are constrained to face in opposite directions. True competitive binding occurs when the epitopes are in close physical proximity. For intermediate sep-
Three patterns of antibody competition observed in this study. Polyclonal antibody was first incubated with immobilized sodium channel protein in a solution phase assay at the indicated dilutions of cell culture supernatants. After washing, one of four monoclonal antibodies was incubated with immobilized sodium channel and bound monoclonal antibody quantitated by the binding of iodinated goat anti-mouse IgG. All experiments were repeated twice with three samples per experimental point. Data are reported as the average of three samples with error bars indicating standard deviation. A, amino-terminal polyclonal antibody I-31 sterically hinders the binding of monoclonal antibodies F/C11 and B/D6 to the interdomain 2-3 region (approximately 900 residues distant from the amino terminus) but has no effect on the binding of two monoclonal antibodies (A/B2 and L/D3) whose epitopes are located immediately adjacent in the amino terminus of the channel. These data support a model in which the amino terminus is in a compact, folded, rigid structure and the 2-3 interdomain region is centrally located. B, binding of I-1771 inhibits subsequent A/B2 and L/D3 binding with a steep concentration dependence, suggesting that the near amino- and distal carboxyl termini of the sodium channel are located less than 3.5 nm apart in the native channel.

### Table II

Summary of antibody competition results

|                | I-31  | I-467  | B-30  | R-12  | B-23  | I-1771 |
|----------------|-------|--------|-------|-------|-------|--------|
| N terminus     |       |        |       |       |       |        |
| A/B2 (AA 1-6)  | -     | +      | +     | -     | +     | -      |
| L/D3 (AA 19-24)| +     | -      | -     | -     | -     | -      |
| Interdomain 2-3 region | -     | +      | +     | +     | +     | +      |

A, amino terminus

B, binding of I-1771 peptide to fusion proteins containing different sodium channel intracellular domains. Fusion proteins composed of maltose-binding protein linked to the rSkM1 carboxyl terminus (residues 1593-1840), the rSkM1 interdomain 2-3 region (residues 794-1017), the rSkM2/H1 carboxyl terminus (residues 1791-2018), and the maltose binding protein alone were immobilized on amylose resin and incubated with a synthetic peptide comprising residues 1-30 of the rSkM1 sodium channel amino terminus (I-1-30). This peptide bound specifically and with high affinity only to the rSkM1 carboxyl-terminal fusion protein, providing experimental support for the hypothesis that these two channel segments interact in vivo.

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![Fig. 4. Three patterns of antibody competition observed in this study.](image1)

![Fig. 5. Binding of I-1-30 peptide to fusion proteins containing different sodium channel intracellular domains.](image2)

binding of one antibody that reduce the affinity of another antibody at a remote epitope appear to occur infrequently (18), we are able to place constraints on the organization of the epitopes under study within the roughly 9-nm diameter envelope of the sodium channel protein (see Barchi (19) for a review of channel physical properties).

For most monoclonal-polyclonal pairs, maximum competition decreased specific binding to ~35% of control values even with the highest concentrations of competing antisera. While this could reflect a measuring artifact resulting from a reduction in $k_{on}$ for the second antibody, this is not the case here since binding rates were determined directly and measurements were made under equilibrium conditions. Several other explanations must be considered. First, a portion of the solubilized sodium channel protein used in the binding assay may be denatured during preparation or storage, resulting in the spatial separation of epitopes which otherwise are close together. Alternatively, sodium channel protein may exist in different conformations, only some of which position the epitopes close to one another. Finally, variable post-translational modification of the sodium channel protein (e.g., phosphorylation) may prevent quantitative monoclonal antibody binding.

It is possible that some of the purified channel protein may be sufficiently denatured to allow separation of epitopes, even though we have shown in the past that most remain functional (16, 17). However, since some monoclonal-polyclonal pairs produce greater binding inhibition (>80%), it is more likely that
the lower levels of inhibition seen with other pairs simply reflect incomplete block as expected for intermediate separated epitopes where multiple polyclonal antibody molecules with varying affinities may need to bind in order to completely occlude the second epitope. Finally, we have no data that addresses the possible role of alternate channel conformations or variable post-translational modification in this process.

Fig. 6. Cartoon depicting our model of sodium channel cytoplasmic domain structure. The figure on the left reflects the view obtained from inside the cell, looking up at the portions of the sodium channel protein which extend into the cytoplasm. The figure on the right represents a side view of the sodium channel protein. Specific points to observe include: (a) the interaction of the carboxyl terminus (lightest shade of gray) with the arch-shaped amino terminus (darkest shade of gray), presenting a face extending away from the bulk of the intracellular mass of the channel; (b) the centrally located interdomain 2–3 region with a fixed orientation to the remainder of the channel’s intracellular segments; (c) the relative organization of each antibody epitope in three-dimensional space. See text for details.

Predictions Concerning the Interdomain 2–3 Region—Several pieces of data suggest a rigid structure for the amino terminus (Fig. 6). These include: 1) the absence of competition between I-31 and either A/B2 nor L/D3 despite the fact that all three epitopes are located within the first 46 residues of the channel sequence; 2) the lack of competition between A/B2 and all site-directed polyclonal antibodies except I-1771 (see below); and 3) the relative resistance of the amino terminus to exogenous proteolysis (8).

Our data support a model in which the first 46 amino acids of the amino terminus forms an arc, with the A/B2 epitope (residues 1–6) facing away from, the L/D3 epitope (residues 19–24) facing partly toward, and the I-31 epitope (residues 31–46) facing directly toward the centrally located interdomain 2–3 region (Fig. 6). Evidence supporting this hypothesis includes the absence of interaction between A/B2 and all polyclonal antibodies except I-1771, the partial interaction of L/D3 with B-30 (interdomain 2–3) and B-23 (beginning of carboxyl terminus), and the interaction between I-31 and monoclonal antibodies F/C11 and F/D6 (interdomain 2–3 region).

Predictions Concerning the Amino Terminus—Several pieces of data suggest a rigid structure for the amino terminus (Fig. 6). These include: 1) the absence of competition between I-31 and either A/B2 nor L/D3 despite the fact that all three epitopes are located within the first 46 residues of the channel sequence; 2) the lack of competition between A/B2 and all site-directed polyclonal antibodies except I-1771 (see below); and 3) the relative resistance of the amino terminus to exogenous proteolysis (8).

Our data support a model in which the first 46 amino acids of the amino terminus forms an arc, with the A/B2 epitope (residues 1–6) facing away from, the L/D3 epitope (residues 19–24) facing partly toward, and the I-31 epitope (residues 31–46) facing directly toward the centrally located interdomain 2–3 region (Fig. 6). Evidence supporting this hypothesis includes the absence of interaction between A/B2 and all polyclonal antibodies except I-1771, the partial interaction of L/D3 with B-30 (interdomain 2–3) and B-23 (beginning of carboxyl terminus), and the interaction between I-31 and monoclonal antibodies F/C11 and F/D6 (interdomain 2–3 region).

We have shown that A/B2 and L/D3 demonstrated competition with each other for binding to immobilized sodium channel protein (11). However, unlabeled F/C11 competed with neither A/B2 nor L/D3 while unlabeled B/D6 demonstrated partial competition with A/B2 and L/D3. No competition was observed between labeled F/C11 and unlabeled B/D6. These data are consistent with our topologic model of the sodium channel amino terminus and interdomain 2–3 regions.

The absence of competition between monoclonals to two adjacent interdomain 2–3 epitopes (F/C11 and B/D6) indicates that these two epitopes are oriented in different directions and, although separated by approximately 100 residues, may have a restricted range of motion. The observation that this region is the most sensitive to proteolysis of the interdomain regions supports a model in which the amino-terminal half of the interdomain 2–3 region extends into the cytoplasm, away from the membrane embedded domains (Fig. 6). The partial inhibition by A/B2 and L/D3 on B/D6 binding, the absence of inhibition by these two monoclonal antibodies on either B-30 or F/C11 binding, and the competition between I-31 and both F/C11 and B/D6 all suggest that the B/D6 epitope (located at the carboxyl-terminal end of the interdomain 2–3 region) is oriented toward and/or closer to the amino terminus than are the F/C11 or B-30 epitopes (located at or just beyond the midpoint of the interdomain 2–3 region) (Fig. 6). Testing these structural hypotheses will require further dissection of cytoplasmic domain topology using competition studies with antibody Fab fragments, physical probes such as fluorescence energy transfer, and direct imaging techniques such as electron diffraction.

Binding of Amino to Carboxyl Terminus—Our data suggest that the channel amino and carboxyl termini are closely interrelated in the tertiary structure of the channel. In order to accommodate this interaction, we suggest a model in which domains 1 and 4 are adjacent in the tertiary structure. This is consistent with previous binding studies which demonstrated competition between α-scorpion toxin and site-specific antibodies directed against the S5-S6 regions of domains 1 and 4 in the rat brain sodium channel (20). Although the functional significance of this interaction is unknown, binding between the amino and carboxyl termini could play a role in channel assembly or in stabilization of channel tertiary structure. Another possibility is suggested by our previous finding that A/B2 and L/D3 differentially label channels in the surface and T-tubular membranes of fast and slow skeletal muscle fibers (21, 22). It is possible that the rigid surface formed by the interaction of amino- and carboxyl-channel segments creates a binding site for cytoskeletal proteins that contribute to the subcellular localization of channels in different membrane environments (12).

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