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Localization of Epitopes for Antibodies that Differentially Label Sodium Channels in Skeletal Muscle Surface and T-Tubular Membranes

Sidney A. Cohen† and Robert L. Barchi‡

†Cardiology Section, Department of Medicine, and the ‡David Mahoney Institute of Neurological Sciences, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Summary. We previously characterized two monoclonal antibodies, A/B2 and L/D3, that bind to the amino-terminus of the sodium channel but produce distinct immunocytochemical patterns in innervated adult skeletal muscle. Because these findings suggested the presence of several channel isoforms, we sought to define the epitopes for each antibody. Five peptides encompassing the amino-terminal 126 residues of the adult skeletal muscle sodium channel were synthesized and tested by radioimmunoassay against each antibody. Both monoclonals bound only to a peptide comprising residues 1–30 (I^{1-30}). A nested set of peptides within this region was then synthesized and used to compete for antibody binding to I^{1-30}. L/D3 binding was quantitatively inhibited by oligopeptides 1–30, 7–30, 13–30, and 19–30 but not 25–30, while binding of A/B2 was blocked only by the intact I^{1-30} peptide. This data implies that the epitope for L/D3 lies within residues 19–25 while the epitope for A/B2 is contained within residues 1–6. These tentative epitope localizations were confirmed using both proteolytic cleavage of I^{1-30} and immunoreactivity of a peptide corresponding to residues 1–12 with A/B2 but not L/D3. Therefore, epitopes for each monoclonal antibody are present in the SkM-1 sequence and are in close proximity in the amino-terminus of the protein. Their characteristic immunocytochemical labeling patterns may reflect differing accessibility of the epitopes in various membrane environments.

Key Words ion channels · channel subtypes · epitope localization · sarcolemma · monoclonal antibodies · radioimmunoassay

Introduction

The voltage-dependent sodium channel protein is responsible for the rapid upstroke of the action potential in most excitable membranes. This channel has been cloned and sequenced from a number of species and tissues (Noda et al., 1984, 1986; Salkoff et al., 1987; Auld et al., 1988; Joho et al., 1988; Kayano et al., 1988; Rogart et al., 1989; Trimmer et al., 1989; Kallen et al., 1990). All of these sodium channels are closely related, with four regions of internal sequence homology, each containing six to eight transmembrane segments including a positively charged amphipathic helix (S_4) hypothesized to be important in channel gating.

Several tissues express two or more sodium channel isoforms that differ slightly in their primary amino acid sequence and are encoded by distinct genes. Two such isoforms have been cloned from skeletal muscle: a tetrodotoxin-sensitive form (SkM-1) found in adult muscle and a tetrodotoxin-resistant form (SkM-2) characteristic of denervated adult and developing skeletal muscle (Trimmer et al., 1989; Kallen et al., 1990).

The existence of additional sodium channel isoforms in innervated adult skeletal muscle has been suggested on the basis of electrophysiologic, immunocytochemical, and toxin binding studies (Jaimovich et al., 1982, 1983, 1986; Barhanin et al., 1984; Arispe et al., 1988). Recently, we used monoclonal antibodies raised against purified muscle sodium channel protein to identify several distinct immunoreactive forms of the channel in adult innervated rat skeletal muscle (Haimovich et al., 1987). One group of monoclonal antibodies (typified by L/D3) labeled only sodium channels in the surface membrane of both fast and slow twitch skeletal muscle fibers while a second group (typified by A/B2) also prominently labeled channels in the T-tubular system of slow twitch fibers. Neither antibody labeled the T-tubular system of fast twitch fibers. The origin of these immunocytochemically identified isoforms has been unclear, however, since only one sodium channel message (SkM-1) has been found in innervated adult skeletal muscle.

Binding studies using metabolically labeled antibodies have demonstrated competition between members of these two antibody groups, suggesting that both epitopes are in the same region of the channel tertiary structure (Casadei & Barchi, 1987). Studies using both endogenous and exogenous pro-

teolysis indicate that both epitopes lie within the amino-terminal 12 kDa of the sodium channel protein (Kraner, Yang & Barchi, 1989; Zwerling, Cohen & Barchi, 1991). In an effort to further define the nature of these immunologically distinct channel forms, we have now mapped the locations of these two epitopes within the SkM-1 adult skeletal muscle sodium channel primary sequence.

Materials and Methods

Materials

All chemicals, enzymes, and chromatographic media were obtained from Sigma (St. Louis, MO). Falcon 3911 MicroTest III microtitre plates were obtained from VWR Scientific (Bridgeport, NJ). Rabbit anti-mouse IgG was obtained from Organon Teknika (Durham, NC). Immunologic grade ^{125}I-Protein A was obtained from Amersham (Irvine, CA). Centricon-100 microconcentrators were obtained from Amicon (Beverly, MA).

Monoclonal Antibodies

The monoclonal antibodies A/B2 and L/D3 were generated against purified rat skeletal muscle sodium channel as previously described (Casadei et al., 1984). Antibodies were isolated from supernatants of hybridoma cell lines grown in Kennett's Hy medium (Casadei & Barchi, 1987).

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 high performance liquid chromatography (HPLC) using a C18 column on an Applied Biosystems 130A microbore HPLC system. Appropriate amino acid composition was confirmed for all peptides. Nested oligopeptides comprising portions of the I^{1-30} peptide were obtained by removing resin containing nascent peptide at different stages during synthesis of the I^{1-30} peptide.

Assays of Antibody Binding

For radioimmunoassays, microtitre plates were precoated with poly-L-lysine in PBS. Peptides (1 μM in PBS) were immobilized to the coated wells by incubating for two hours at room temperature. Unbound sites were then blocked with 4% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Hybridoma supernatants were diluted in 2% BSA/PBS and incubated with immobilized peptide for 2 hr. After washing with 0.05% Tween-20 in PBS, rabbit anti-mouse IgG diluted 1 : 1000 in 2% BSA/PBS was used as a bridging antibody prior to incubation with ^{125}I-Protein A (1.25 μCi/ml). After extensive washing, immobilized ^{125}I-Protein A was quantitated using an LKB Model 1270 gamma counter. Competition assays were performed by preincubating 1 : 10 dilutions of hybridoma supernatants overnight with the indicated concentrations of oligopeptides in 2% BSA/PBS prior to use for radioimmunoassay. All radioimmunoassays were performed in duplicate and each experiment was repeated at least three times.

Proteolysis of the I^{1-30} Peptide

Reactions with the I^{1-30} peptide were carried out at room temperature. The conditions were as follows: L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Type XIII), 7.5 units/ml in PBS (pH 7.4) for 2 hr; V8-protease (Type XVII-B), 60 units/ml in PBS (pH 7.8) for 16 hr; collagenase (Type VII), 500 units/ml in 50 mM HEPES (pH 7.5) and 5 mM $CaCl_2$ for 16 hr; cathepsin C (Type X), 3 units/ml in 50 mM ammonium carbonate (pH 6.0) for 1 hr; and leucine aminopeptidase (Type VII), 10 units/ml in 50 mM $NaPO_4$ (pH 8.0) for 30 min. Reactions were terminated by addition of 100 mM EDTA for collagenase, 10 μM PMSF for trypsin and V8-protease, and by separation of enzyme from substrate and products by ultrafiltration through Centricon-100 microconcentrators at 1,000 × *g* for leucine aminopeptidase and cathepsin C. Samples were diluted to 1 μM peptide in PBS (pH 7.4) before use in radioimmunoassay. Proteolysis was monitored by size exclusion chromatography under denaturing conditions on G-25 Sephadex resin. Under the above conditions, all enzymes produced a reduction in size of the intact peptide with the simultaneous appearance of an additional peptide fragment.

Results

Monoclonal antibodies A/B2 and L/D3 both identify a comparable diffuse 276 kDa band on immunoblots of either crude skeletal muscle protein or purified muscle sodium channel (Casadei et al., 1984; Kraner, Tanaka & Barchi, 1985; Casadei, Gordon & Barchi, 1986). Proteolysis studies on the sodium channel protein have localized the epitopes for both antibodies to within a region encompassing ~12,000 Da of the amino terminus of the channel (Kraner et al., 1989; Zwerling et al., 1991). Based on these previous studies, we focused our efforts to further localize these epitopes on the first 126 amino acids (14,131 Da) of the SkM-1 sequence.

Five partially overlapping peptides, which together comprise most of the amino-terminal 126 amino acids of the SkM-1 sequence, were first synthesized and tested by radioimmunoassay (RIA) for binding to A/B2 and L/D3. Both monoclonal antibodies bound to a peptide corresponding to the first 30 amino acids of the channel primary sequence (I^{1-30}) but demonstrated no immunoreactivity with any of the other peptides (Fig. 1).

A nested set of peptides within residues 1 to 30 sharing a common carboxy terminus were then constructed. The oligopeptides synthesized encompassed amino acids 25–30 (I^{25-30}), 19–30 (I^{19-30}), 13–30 (I^{13-30}), and 7–30 (I^{7-30}). Because small peptides like these do not quantitatively bind to RIA plates,

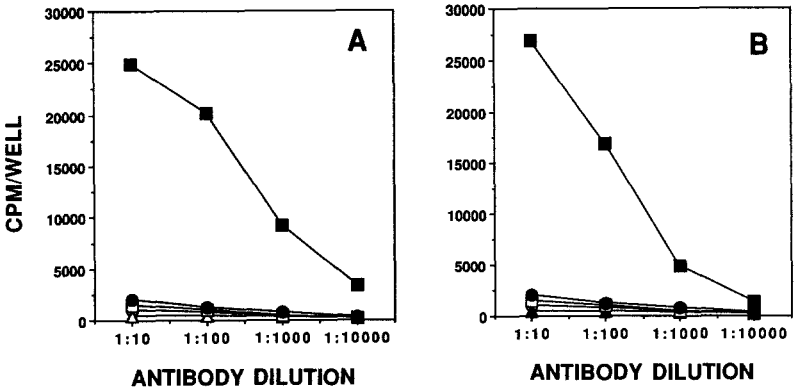

Fig. 1. Binding of monoclonal antibodies A/B2 (*A*) and L/D3 (*B*) to oligopeptides corresponding to segments of the skeletal muscle sodium channel amino terminus. The results of solid-state radioimmunoassays are shown. Microtitre plates were precoated with poly-L-lysine and incubated with 1 μM of either BSA or peptides comprising amino acids 1–30 (■), 31–49 (○), 57–75 (△), 74–101 (□), 97–126 (●) of the adult skeletal muscle sodium channel sequence. Unbound sites were blocked with 4% (wt/vol) BSA in PBS prior to incubation with increasing dilutions of the indicated antibody in 2% BSA in PBS. Bound antibody was quantitated by gamma counting following incubation with ^{125}I-Protein A. Both monoclonal antibodies bound specifically only to the peptide comprising amino acids 1–30 (I^{1-30}).

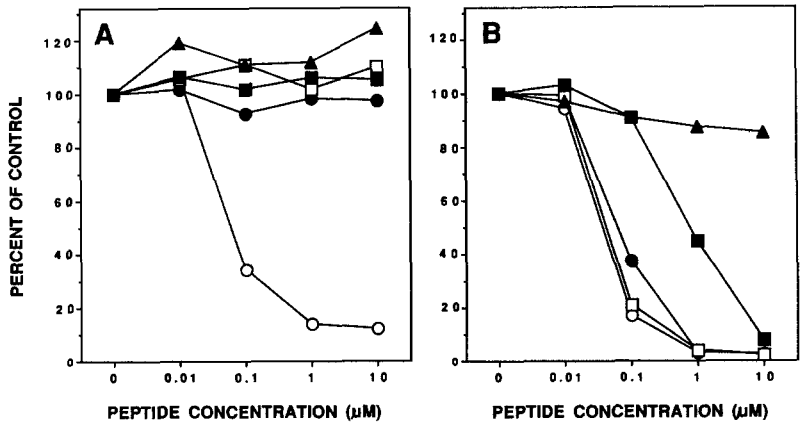

Fig. 2. Competition for binding of A/B2 (*A*) or L/D3 (*B*) to I^{1-30} produced by preincubation of the monoclonal antibodies with various oligopeptides. 1 : 10 dilutions of hybridoma supernatants were incubated overnight with the indicated concentration of oligopeptides in the presence of 2% BSA in PBS prior to application to RIA plates and quantitation of bound antibody. Oligopeptides used included I^{25-30} (▲), I^{19-30} (■), I^{13-30} (□), I^{7-30} (●), and I^{1-30} (○). Only I^{1-30} was able to competitively inhibit A/B2 binding to immobilized I^{1-30}. L/D3 binding was competitively inhibited by oligopeptides I^{1-30}, I^{7-30}, I^{13-30} and I^{19-30} but not I^{25-30}.

antibody binding to these fragments was evaluated by competition assays in which antibodies were preincubated with increasing concentrations of each partial peptide before being used in radioimmunoassays against the immobilized I^{1-30} peptide.

Binding of L/D3 to I^{1-30} was quantatively inhibited by preincubation with partial peptides I^{7-30}, I^{13-30}, and I^{19-30} but not I^{25-30}, while A/B2 binding to I^{1-30} was blocked only with the intact I^{1-30} peptide (Fig. 2). These results suggest that the epitope for L/D3 encompasses amino acids 19–25 while the epitope for A/B2 lies within amino acids 1–6. In order to confirm these epitope localizations, a series of proteolysis experiments was then undertaken.

Five proteases with differing sequence specificities were tested for their ability to proteolyze the I^{1-30} peptide and disrupt monoclonal antibody binding. The extent of proteolysis produced by each enzyme was monitored by size exclusion chromatography on G-25 Sephadex resin in which a reduction in size of the intact peptide and the appearance of additional peptide fragments could be monitored. Cathepsin C removes amino-terminal dipeptides until blocked by a penultimate proline, although the emergence of the sequence *X-X-Pro-X* has also been reported to terminate enzyme activity (McDonald et al., 1967, 1974). Leucine aminopeptidase cleaves single amino acids from the amino terminus until blocked by proline (Pfleiderer, 1970; Nagasawa, Nagasawa & Heinrich, 1980; Hasegawa, Kodama & Akatsuka, 1985). Both cathepsin C and leucine aminopeptidase, enzymes predicted to cleave no further than amino acids 10 and 6, respectively, rapidly eliminated A/B2 binding without affecting L/D3 binding (Fig. 3).

V-8 protease (predicted cleavage on the carboxy-terminal side of Glu^{23} and Glu^{29}) quickly destroyed L/D3 binding to the I^{1-30} peptide but had no effect on A/B2 binding (Fig. 3). Based on the appearance of peptide fragments on G-25 Sephadex

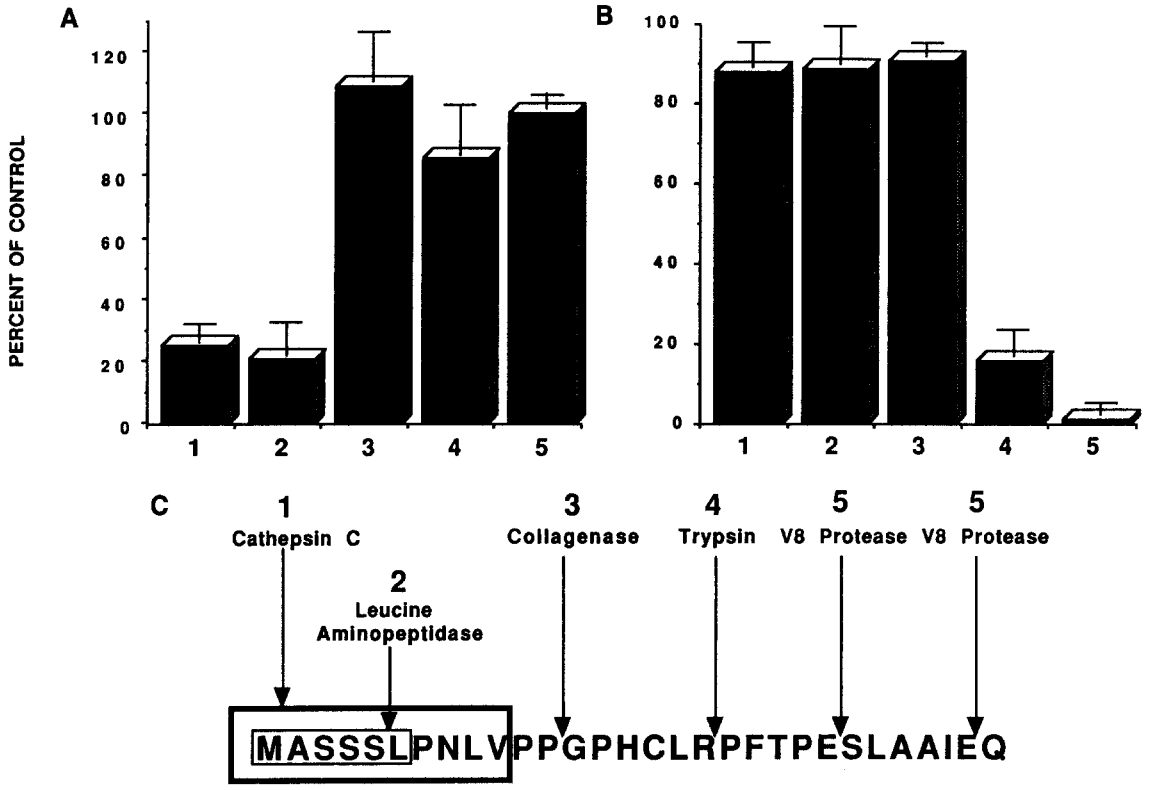

Fig. 3. Effect of proteolysis on the antigenicity of I^{1-30} as determined with A/B2 (*A*) and L/D3 (*B*) with an illustration depicting the sites of proteolysis on the I^{1-30} peptide (*C*). I^{1-30} was incubated with leucine aminopeptidase (*1*), cathepsin C (*2*), collagenase (*3*), TPCK-treated trypsin (*4*), or V8-protease (*5*) under the conditions detailed in Materials and Methods. All values (± SD) are the average of at least three separate determinations. RIA's were performed as described in Fig. 1 except that proteolyzed I^{1-30} was used in place of intact I^{1-30}. Only cathepsin C and leucine aminopeptidase destroyed the A/B2 epitope, consistent with the localization of this epitope of the amino-terminal portion of the I^{1-30} peptide. These two enzymes and collagenase had no effect on the L/D3 epitope, while V8-protease and trypsin appeared to destroy the L/D3 epitope, confirming its localization to the carboxy-terminal portion of I^{1-30}.

chromatography, cleavage at amino acid 23 was detected but a cut at amino acid 29 could not be documented.

Trypsin (predicted cleavage on the carboxy-terminal side of Arg^{18}) had no effect on A/B2 binding but produced a marked decrease in L/D3 binding (Fig. 3). Competition studies were then performed in which L/D3 was incubated with increasing concentrations of trypsin-cleaved I^{1-30} peptide before being applied to intact I^{1-30} peptide immobilized on the RIA plate (Fig. 4*A*). While the cleaved peptide was able to adsorb all of the L/D3 antibody, the affinity of antibody binding for this peptide was reduced >20-fold ($K_{0.5}$ shifted from 0.075 to 1.5 mM). This reduced affinity matches that observed in the competition studies performed with the partial peptide I^{19-30} (Fig. 2).

Collagenase, which cuts the sequence Pro-*X*-cut-Gly-Pro where *X* is a neutral amino acid and should cleave the I^{1-30} peptide between Pro^{11}-Pro^{12} and Gly^{13}, had no effect on A/B2 or L/D3 binding (Fig. 3).

Finally, in order to confirm that the antigenic determinant for A/B2 is confined to the amino-terminal portion of the I^{1-30} peptide, a peptide comprising the first 12 amino acids was synthesized and tested in a competition assay against the intact I^{1-30} peptide (Fig. 4*B*). A/B2 antisera bound this peptide with greater affinity than the I^{1-30} peptide itself, confirming the presence of the antigenic determinate for A/B2 within this sequence. No binding of L/D3 to this peptide was seen.

Discussion

We have mapped the epitopes for two monoclonal antibodies that differentially bind to sodium channel protein in surface and T-tubular membranes when used in immunocytochemical studies of adult innervated rat skeletal muscle. Based on immunoreactivity in radioimmunoassays against five partially overlapping peptides comprising most of the amino-terminal 14,131 Da of the SkM-1 sequence, the epi-

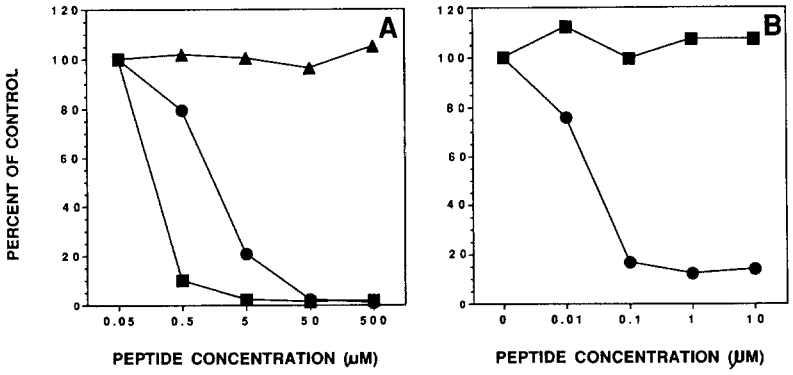

Fig. 4. (*A*) Competition of L/D binding to the I^{1-30} peptide by trypsin-cleaved I^{1-30}. I^{1-30} was cleaved with trypsin as described in Materials and Methods. L/D3 was pre-incubated with the cleaved I^{1-30} peptide prior to being applied to the RIA plate containing immobilized intact I^{1-30} (●). Intact I^{1-30} was used as a positive control (■) and BSA was used as a negative control (▲). While the trypsin-cleaved peptide was able to adsorb all of the L/D3 antibody, the affinity of antibody binding for this peptide was reduced >20-fold. This reduced affinity matches that observed in the competitive inhibition studies performed with the partial peptide I^{19-30} (Fig. 2*B*). (*B*) Inhibition of A/B2 and L/D3 binding to the intact I^{1-30} peptide by an oligopeptide comprising amino acids 1–12 (I^{1-12}). 1 : 10 dilutions of antibodies A/B2 (●) and L/D3 (■) were incubated overnight with the indicated concentrations of peptide I^{1-12} in 2% BSA in PBS prior to application to intact I^{1-30} peptide immobilized on RIA plates. A/B2 but not L/D3 bound specifically to this peptide, confirming the location of the A/B2 epitope to within the first 12 amino acids of the adult skeletal muscle sodium channel sequence.

topes for monoclonal antibodies A/B2 and L/D3 were first localized to the amino-terminal 30 amino acids of the SkM-1 sequence. Finer resolution was then achieved for both epitopes using both nested peptides within I^{1-30} and proteolysis of the I^{1-30} peptide. Our data with the nested peptides localized the epitope for A/B2 within the first six amino-terminal residues in the SkM-1 sequence; subsequent proteolysis studies with sequence-specific enzymes supported this epitope localization. Localization of the epitopes to this sequence was then confirmed by radioimmunoassay using the I^{1-12} peptide. Interestingly, A/B2 binds with higher affinity to I^{1-12} than to either I^{1-30} or purified sodium channel protein.

The epitope for L/D3 was localized to the region including amino acids 19–24. L/D3 binds with comparable affinity to the I^{1-30}, I^{7-30}, and I^{13-30} oligopeptides and to purified sodium channel protein but with reduced affinity to both the I^{19-30} oligopeptide and to the trypsinized I^{1-30} peptide. This reduced affinity suggests that while the I^{19-30} peptide contains the L/D3 epitope, portions of the sequence amino-terminal to Pro^{19} may constrain the peptide to conformations better recognized by antibody. Local secondary structure imposed by Cys^{16} and the six proline residues in the first 24 amino acids of the SkM-1 sequence may contribute to both the protease resistance (Zwerling et al., 1991) and immunoreactivity of this region (Fig. 5).

Based on the above studies, we conclude that the epitopes for both A/B2 and L/D3 are present in the SkM-1 sequence and are located in close proximity within the first 24 amino acids of the primary sequence. Neither of these epitopes are conserved in the rat skeletal muscle TTX-insensitive sodium channel (SkM-2) (Kallen et al., 1990) or in the rat brain sodium channels (Noda et al., 1986: Kayano et al., 1988; Ahmed et al., 1990).

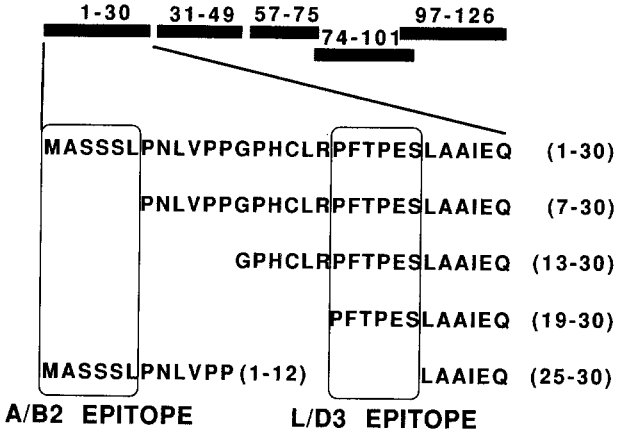

Fig. 5. Summary of epitope localization for the A/B2 and L/D3 epitopes based on analysis of binding to synthetic oligopeptides.

The differential binding patterns exhibited by these two antibodies in immunocytochemical studies of adult muscle must now be re-evaluated in light of these findings. These patterns could be due to structural heterogeneity at one or both epitopes or to differing accessibility to these epitopes in a manner that varies with intracellular location. Sub-

tly different forms of the sodium channel protein, resulting from amino-terminal proteolysis, mRNA splicing variants, or post-translational modification could be present. Several lines of evidence suggest that amino-terminal proteolysis is unlikely. Both antibodies identify bands of identical apparent molecular weight on immunoblots of both skeletal muscle protein and purified sodium channel protein (Casadei et al., 1984, 1986; Kraner et al., 1985, 1989). In the intact channel, both epitopes are highly resistant to proteolysis (Zwerling et al., 1991). The fact that A/B2, which recognizes the most distal epitope, identifies the broadest range of channels immunocytochemically also argues against selective cleavage as the cause of immunogenic diversity. Finally, in competition studies using metabolically labeled antibodies, unlabeled L/D3 was able to nearly completely inhibit [^{35}S]-labeled A/B2 binding to purified sodium channel protein (Casadei & Barchi, 1987), indicating that all channels that bind A/B2 also contain the L/D3 epitope.

Another possible structural explanation would be the presence of splicing variants at the RNA level. While no evidence for alternative splicing has been found for mammalian sodium channels with the possible exception of rat brain II and IIa (Ahmed et al., 1990), alternative splicing has been shown to produce distinct sodium channel subtypes with dissimilar phenotypes in *Drosophila* (Ganetzky & Loughnex, 1989; Loughney, Kreber & Ganetsky, 1989). To date, however, we have found no evidence of alternatively spliced mRNA variants of SkM-1 in adult innervated skeletal muscle. Furthermore, competition experiments argue strongly that the A/B2 and L/D3 epitopes are both present on the same protein and hence must both be represented in the same mRNA (Casadei et al., 1984).

Different channel isoforms could also be produced by post-translational modification. While the sequence encompassing the A/B2 epitope does not contain consensus sequences for protein phosphorylation or N-glycosylation, a consensus phosphorylation sequence is present within the region containing the L/D3 epitope. Although previous studies with SkM-1 indicate that this site is not phosphorylated by cAMP-dependent protein kinase (Yang & Barchi, 1990; S.A. Cohen and R.L. Barchi, *unpublished observations*), phosphorylation under other conditions is possible. However, the nearly quantitative inhibition of metabolically labeled A/B2 binding by unlabeled L/D3 makes this explanation unlikely.

An alternate explanation for these findings is the differential interaction of regions of the channel alpha subunit containing the monoclonal epitopes with membrane cytoskeletal elements, channel beta subunits, or other associated proteins. The adult skeletal muscle sodium channel is noncovalently associated with a heavily glycosylated beta subunit (Roberts et al., 1986; Roberts & Barchi, 1987). Little information exists concerning the molecular interactions of alpha and beta subunits except that, in adult skeletal muscle, they are associated with a 1 : 1 stoichiometry. Differential reactivity with intracellular portions of the beta subunit or with different beta subunits in a manner that varies with intracellular location is one possible explanation for the immunocytochemical findings.

A second possible explanation involves differential interaction of the alpha subunit with membrane cytoskeletal elements or other intracellular proteins in a location-specific manner. Several lines of evidence indicate that lateral mobility is constrained for sodium channel protein in certain parts of nerve and muscle cells (Stuhmer, Stanfield & Almers, 1982; Angelides et al., 1988). In addition, several authors have provided biochemical evidence for the association of ankyrin and spectrin with CNS sodium channel protein (Srinivasan et al., 1988; Wood & Angelides, 1989; Kordeli et al., 1990). Finally, evidence has been presented for the direct interaction of sodium channels with G proteins (Schubert et al., 1989; Brown & Birnbaumer, 1990). Despite accumulating experimental evidence, the actual sites of molecular interaction have yet to be defined. Future studies will be directed at localizing these sites of interaction and distinguishing which of the above explanations are responsible for the differential labeling seen in immunocytochemical studies.

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