Topological Localization of a Segment of the Eel Voltage-Dependent Sodium Channel Primary Sequence (AA 927–938) That Discriminates Between Models of Tertiary Structure

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Antibodies were raised against a synthetic peptide corresponding to residues 927–938 of the eel electroplax sodium channel primary sequence. This segment, lying between putative internal repeat domains II and III, is postulated to be exposed on the cytoplasmic surface of the membrane in several recent models of channel tertiary structure and on the external surface in another. The antiserum and affinity-purified IgG derived from it specifically recognize the peptide and the eel sodium channel in a solid-phase radioimmunoassay and bind to a single diffuse band of 260–280 kDa on Western blots of eel electroplax membrane proteins. All reactions are blocked by co-incubation of the antibodies with the synthetic peptide (1 μ M). The antibody immunoprecipitates the solubilized channel in a form that retains its characteristic high-affinity binding of saxitoxin. In eel electroplax, the antibodies label only the innervated membrane known to contain sodium channels; at the ultrastructural level, this labeling is exclusively associated with the cytoplasmic surface of the membrane. Sodium channels containing the epitope are not seen in the postsynaptic membrane or in the membrane of the presynaptic nerve terminal.

Segment 927–938 of the eel sodium channel is accessible on the surface of the protein in its solubilized form and is exposed in the cytoplasmic face of the innervated membrane of the electroplax *in situ*. This location is consistent with 3 models of channel structure but not with a fourth.

The voltage-dependent ion channel that controls membrane permeability to Na⁺ during the generation of an action potential has been isolated and characterized from rat, rabbit, and eel (see Barchi, 1988, for review). In each case, the channel contains a large glycoprotein component of ~260 kDa (Agnew et al., 1980; Hartshorne and Catterall, 1981; Kraner et al., 1985). The channel from mammalian nerve and muscle may also contain 1 or 2 smaller subunits of 30–40 kDa (Casadei et al., 1986; Messner and Catterall, 1986; Roberts and Barchi, 1987); however, the 260 kDa component appears to include all the mo-

lecular machinery necessary for voltage-dependent activation, inactivation, and cation selectivity (Noda et al., 1986b; Stuhmer et al., 1987).

The primary sequences for the eel sodium channel and for the 260 kDa component of 2 forms of the sodium channel from rat brain have been determined (Noda et al., 1984, 1986a). All of these proteins contain 4 large internal repeat domains of ~275 amino acids each, which are themselves homologous and may have arisen from gene duplication. Six to 8 putative transmembrane helices can be identified within each domain, including one strongly amphipathic helix with a positive charge at every third residue. The sequences of these channel proteins are highly homologous, with >60% of eel channel residues within the 4 internal repeat domains either identical or conservatively substituted in the comparable segments of the rat channels (Noda et al., 1986a).

Based on primary sequence data, a number of models have been proposed for the organization of the transmembrane helices and for the tertiary structure of the channel protein (Greenblatt et al., 1985; Kosower, 1985; Guy and Seetharamulu, 1986; Noda et al., 1986a). These models vary in the number of transmembrane segments they contain and in the relative orientation of helices within the membrane, as well as in the potential physiological function ascribed to various regions of the structure. In order to differentiate between such folding schemes and proposed structures, independent probes of channel topography are needed. Antibodies raised against synthetic peptides corresponding to known segments of the protein primary sequence can prove effective in this regard.

Previously we have used this approach to localize the C-terminal portion of the eel sodium channel to the cytoplasmic side of the electroplax membrane (Gordon et al., 1987). In this report, a similar approach is used to localize a portion of the central region of the protein that is hypothesized to be intracellular in several models and extracellular in another.

Materials and Methods

Peptide synthesis. A peptide designated R-6, corresponding to amino acids 927–938 of the eel electroplax sodium channel primary sequence (Noda et al., 1984), with the addition of a cysteine residue at the carboxy terminus, was synthesized manually using modifications of the solid-phase methods described by Barany and Merrifield (1980). The support resin was chloromethylated polystyrene esterified with Boc-*s*-*p*-methoxybenzyl-L-cysteine (Peninsula Laboratories). The completed peptide was cleaved from the resin with liquid hydrogen fluoride at 0°C, extracted with 5% acetic acid in water, and lyophilized.

The crude peptide was dissolved in 5% acetic acid in water and purified by reverse-phase chromatography on a C₁₈ column using a

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Figure 1. Radioimmunoassay of antiserum raised against peptide R-6 (●), affinity-purified IgG prepared from this serum (■), and normal rabbit serum (▲), each measured against constant amounts of immobilized R-6 peptide. Affinity-purified IgG exhibited a level of immunoreactivity comparable to the antiserum but at IgG concentrations 100- to 1000-fold lower.

gradient of 0–90% acetonitrile in 0.05% trifluoroacetic acid. Amino acid analysis was carried out on samples hydrolyzed for 24, 48, or 96 hr in 6 N HCl. For quantitation of cysteine, samples were oxidized with performic acid for 4 hr at 0°C prior to hydrolysis. Sequence analysis was performed on an Applied Biosystems model 470A gas-phase microsequencer.

For the production of antibodies, the purified peptide was coupled to soybean trypsin inhibitor (~5 mol peptide/mol protein) through the C-terminal cysteine using *N*-succinimidyl-3-(2-pyridyldithio)propionate. New Zealand White rabbits were immunized with the coupled protein as previously described (Gordon et al., 1987).

Preparation of eel sodium channel. A fraction enriched in sodium channels was prepared from eel electroplax membranes according to the method of Miller et al. (1983) with some modifications. Electroplax membranes were prepared by differential centrifugation after homogenization of the tissue in buffer containing 50 mM potassium phosphate (pH 7.4), 5 mM EDTA, 5 mM EGTA, 0.1 μg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM iodoacetamide (buffer A). Membranes were then solubilized with 1% Lubrol-PX in buffer A. The solubilized channel was adsorbed to DEAE-Sephadex A-25 and washed extensively in buffer A containing 0.2 M NaCl and 0.1% Lubrol supplemented with egg phosphatidyl choline (Sigma) (7:1 detergent:lipid molar ratio; Lubrol-PC). A fraction containing the sodium channel was eluted with buffer A containing 0.1% Lubrol-PC and 0.6 M KCl, concentrated by ultrafiltration on an Amincon YM-100 filter, diluted 1:4 with 20 mM HEPES (pH 7.4) and 0.1% Lubrol-PC, and reconcentrated to the original volume. The final solution, containing 150–200 pmol sodium channel/ml, was frozen at –78°C until needed. The concentration of sodium channels was determined using ³H-saxitoxin binding (Kraner et al., 1985).

Solid-phase radioimmunoassay. Radioimmunoassays (RIAs) were performed in microtiter plates as previously described (Gordon et al., 1987). For assays with immobilized peptide, wells were coated with 50 pmol of peptide in PBS (pH 7.4). For assays with sodium channel protein, wells were first coated with 0.1% poly(L-lysine) in PBS and then incubated with 50 μl aliquots of the eel electroplax DEAE fraction containing 7.5–10 pmol sodium channel. For competition studies with antibody binding, increasing concentrations of free peptide were added to the antibody solution prior to the RIA and were present during the 2 hr exposure of the antibody to the immobilized channel or peptide.

Immunoprecipitation. Immunoaffinity-purified IgG at the indicated amounts was mixed with 60 μl aliquots of the DEAE fraction of solubilized eel electroplax membrane proteins containing ~7 pmol of sodium channel in a total reaction volume of 200 μl with 20 mM HEPES (pH 7.4) and 0.1% Lubrol-PC. After incubation for 12 hr at 4°C, 100 μl of a 10% (wt/wt) suspension of *Staphylococcus aureus* cells prepared as per Kessler (1976) was then added to each aliquot and incubated for a further hour at 4°C. Immobilized antibody-antigen complex was col-

Figure 2. Binding of antiserum to immobilized synthetic peptide (●) or immobilized eel sodium channel protein (■) was competitively blocked by co-incubation with the soluble R-6 peptide. In this system, 50% inhibition was seen at peptide concentrations between 0.05 and 0.3 μM. Addition of peptide had no effect on the low level of binding found with normal rabbit serum (▼). For each point, binding is expressed as a percentage of maximal binding determined for the immunoreagent in the absence of soluble peptide.

lected by centrifugation in an airfuge, washed twice in HEPES–Lubrol-PC buffer, and resuspended in 1.4 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 100 mM choline chloride and 0.1% Lubrol-PC. Using rapid filtration (<10 sec) on glass fiber filters (GF/C, Whatman), triplicate 200 μl samples of each suspended pellet were assayed with 15 nM ³H-saxitoxin (STX) plus or minus 1 μM cold TTX to determine specific STX binding.

Immunocytochemistry. Details of the methods used for light and electron microscopic immunocytochemistry have been reported previously (Gordon et al., 1987; Haimovich et al., 1987). Immunofluorescence light microscopy was carried out on frozen sections of the electric organ of *Electrophorus*. Sections were incubated for 30 min in Dulbecco's PBS containing 10% normal goat serum (buffer B), and then for an additional 2 hr in the anti-peptide antiserum (1:1000 dilution in buffer B) with or without 1 μM R-6 peptide or with control preimmune serum in the same buffer. After washing in buffer B, bound IgG was localized with rho-

Figure 3. Immunoprecipitation of sodium channel high-affinity saxitoxin (STX) binding sites by affinity-purified anti-R-6 IgG. Eel membrane proteins were solubilized in Lubrol and incubated with increasing amounts of the antibody. Antigen-antibody complexes were then isolated with *S. aureus* cells, and the resultant immobilized high-affinity STX binding sites were measured with a rapid filtration assay. In the absence of antibody, no specific STX binding was immobilized by the *S. aureus*, while increasing amounts of added antibody immobilized increasing amounts of channel. Channel immobilized is equated to measured specific high-affinity STX binding, defined as total binding of 15 nM ³H-STX minus the nonspecific binding observed in the presence of 1 μM TTX.

Figure 4. Antiserum against R-6 and the affinity-purified IgG derived from it specifically label a single diffuse high-molecular-weight band of 260–280 kDa on Western blots of eel electroplax membrane proteins (**B**). This binding is blocked by preincubation of the antibody with 1 μ M soluble R-6 peptide (**A**). No other labeled bands are seen.

Figure 5. Antibodies against the R-6 peptide exhibit specific immunoreactivity with the innervated face of the eel electrocyte. **A**, Phase-contrast image of an electrocyte in cross section. The upper surface is the noninnervated face (*N*), while the lower surface is the innervated membrane (*I*), which contains the voltage-dependent sodium channel. **B**, Fluorescence image of the same section labeled with antibody against R-6 and a rhodamine-conjugated second antibody, demonstrating immunofluorescence of the innervated membrane only. The anti-R-6 antiserum was used at a 1:1000 dilution. Original magnification, $\times 600$.

damine-conjugated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA).

For electron microscopy, fresh electric organ was dissected on ice into 1 mm³ cubes, fixed for 1 hr at 0°C with 1% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), and then incubated for 12 hr with 10% normal goat serum in 0.1 M sodium phosphate buffer at pH 7.4 (buffer C). Blocks were then washed with buffer C and exposed for 6 hr at 0°C with agitation to immunoaffinity-purified anti-R-6 IgG in buffer C (1:3 dilution of a solution containing ~ 200 μ g total protein). Blocks were washed extensively with repeated changes of buffer C, and then incubated for an additional 6 hr with colloidal gold-labeled goat anti-rabbit IgG (SPI Supplies, West Chester, PA) diluted 1:20 in the same buffer. Blocks were postfixed in 3% (vol/vol) glutaraldehyde in buffer C followed briefly by 1% (wt/vol) osmium tetroxide and processed for electron microscopy.

Results

A synthetic peptide corresponding to residues 927–938 of the eel electroplax sodium channel primary sequence was synthesized with the addition of a cysteine residue at the carboxy terminus to assist in subsequent coupling reactions. The product, designated peptide R-6, was purified by HPLC on a reverse-phase C-18 column. Analysis of the resultant single peak for amino acid composition and sequence confirmed that the peptide was identical to residues 927–938 of the eel protein.

Antibodies were raised in rabbits against the peptide covalently coupled to soybean trypsin inhibitor. Although several

Figure 6. Immunofluorescent labeling of the electrocyte innervated membrane by the anti-R-6 antiserum is blocked by co-incubation of the antiserum with the purified R-6 peptide (1 μ M). *A*, Phase-contrast image of an electrocyte in cross section showing the innervated (*I*) and noninnervated (*N*) membranes. *B*, Fluorescence image of the same section stained with the anti-R-6 antiserum (1:1000 dilution) followed by a rhodamine-conjugated second antibody. The innervated membrane is intensely labeled. *C*, Parallel section stained with anti-R-6 antiserum in the presence of purified peptide (1 μ M). Labeling of the innervated membrane is specifically blocked. Original magnification, $\times 600$.

Figure 7. Localization of affinity-purified anti-R-6 IgG binding sites on the innervated membrane of eel electrocytes at the ultrastructural level. In this preparation, the entire electrocyte can be scanned in cross section at the electron microscopic level from the innervated to the noninnervated face, allowing unequivocal determination of extracellular (ECS) and cytoplasmic aspects of the surface membranes. Colloidal gold-labeled goat anti-rabbit IgG was used to visualize the antipeptide antibody. *A* and *B*, At the innervated membrane, labeling was exclusively restricted to the cytoplasmic surface both in superficial areas and in the deep invaginations of the surface that characterize this membrane. *C* and *D*, No labeling was seen of the noninnervated membrane in the same cells. In these experiments, immunoaffinity-purified anti-R-6 IgG having a total protein concentration of $\sim 200 \mu\text{g}/\text{ml}$ was used at a 1:3 dilution. Scale bars (lower right), $0.5 \mu\text{m}$.

animals generated immune responses, one rabbit produced an antiserum that was chosen for use in all subsequent experiments. This antiserum was capable of recognizing the immobilized peptide in a solid-phase RIA at dilutions in excess of 1:10,000.

Antibodies specific for the R-6 peptide were purified from this antiserum by immunoaffinity chromatography on a Sepharose 4B column containing the covalently bound peptide. Affinity-purified IgG also recognized the immobilized peptide in the solid-phase RIA (Fig. 1), although at IgG concentrations 3 orders of magnitude lower than serum. This material was used for subsequent localization studies at the electron microscopic level.

Binding of antipeptide antibody to the R-6 peptide and to immobilized eel sodium channel was quantitatively blocked by

co-incubation of the antibody with increasing concentrations of the soluble peptide (Fig. 2). For competition with immobilized channel, 50% inhibition was obtained for the antiserum with a soluble peptide concentration of 30–50 nM. Addition of the peptide had no effect on the nonspecific binding observed with normal rabbit serum, and the residual binding remaining with the antiserum at high concentrations of soluble peptide approached that of the normal serum.

Immunoaffinity-purified IgG against the R-6 peptide immunoprecipitated the sodium channel in a mixed micellar form with detergent and phospholipid after solubilization in Lubrol. These immunoprecipitated channels retained sufficient native conformation to exhibit high-affinity STX binding (Fig. 3). In addition, on Western blots of crude eel electroplax membrane

Figure 8. Sodium channels identified by affinity-purified IgG against the R-6 peptide are found along the surface membrane of the innervated face and its invaginations but not in those regions of the membrane immediately below the presynaptic nerve terminals. Also, no labeling is seen in the membrane of the presynaptic nerve terminal itself. *NT*, nerve terminal; *PSM*, postsynaptic membrane; *ECS*, extracellular space. Original magnification, $\times 12,600$.

proteins, both the antiserum against R-6 and the affinity-purified antibody preparation reacted specifically with a single diffuse band centered at ~ 260 kDa that corresponded in location to the sodium channel protein in this preparation (Fig. 4). Again, immunoreactivity was blocked by preincubation of either antibody preparation with soluble peptide. The antiserum showed no reactivity with sodium channel components from rat or rabbit sarcolemma in comparable Western blots and gave no evidence of cross-reactivity with other eel membrane proteins.

At the light microscopic level, antibodies against the R-6 peptide labeled the innervated membrane of frozen sections of eel electroplax cells (Fig. 5) but showed no immunoreactivity with the noninnervated face of these cells, consistent with the known distribution of sodium channels in this tissue. Co-incubation of the anti-R-6 antibody with the purified R-6 peptide ($1 \mu\text{M}$) prior to application to the tissue section completely blocked the intense staining of the innervated membrane seen in the absence of the peptide, confirming the specificity of this staining (Fig. 6).

Colloidal gold-labeled second antibody was then used to localize binding of the affinity-purified antibody at the electron microscopic level in the same tissue. In this preparation, the entire electrocyte can be seen in cross-sectional orientation at the electron microscopic level, allowing unequivocal determination of the cytoplasmic and extracellular aspects of the surface membrane. The anti-R-6 antibody, as identified by the colloidal gold particles, was again found associated only with the innervated membrane, consistent with the results of the immunofluorescence studies at the light microscopic level. Along the innervated membrane the particles were localized exclusively on

the cytoplasmic side both at the cell surface and along the extensive invaginations of this membrane into the superficial cytoplasm of the cell (Fig. 7). This localization was found in each of 3 separate preparations using tissue obtained from different eels.

Since the eel electroplax is a degenerate muscle cell, and since sodium channels are known to be present in high concentrations in the postsynaptic membrane of the neuromuscular junction in frog, snake, and rat skeletal muscle, we also examined synaptic connections in our preparation at the electron microscopic level. No labeling was seen in the immediate postsynaptic membrane of these junctions, although sodium channels which immunoreacted with the anti-R-6 antibody were present in adjacent nonjunctional surface membrane (Fig. 8). This did not appear to be a penetration or labeling artifact since the cytoplasmic face of invaginations of the surface membrane lying immediately below the postsynaptic membrane were fully labeled. In addition, no labeling was associated with the membrane of the presynaptic nerve terminal, suggesting that in eel, as in rat, the sodium channel in nerve and muscle may be antigenically distinct.

Discussion

Although the primary sequence of the eel electroplax sodium channel is now known, models of its tertiary structure are based only on theoretical analyses of these linear sequences (Greenblatt, 1985; Kosower, 1985; Guy and Seetharamulu, 1986; Noda et al., 1986a), and validation of these models will depend on the availability of independent topographical information. Di-

rect localization of known segments of the primary sequence using synthetic oligopeptides and antibodies raised against them as probes can provide this information. With this and related biochemical data, these models can be constrained and refined to provide a more precise approximation of the channel's true structure.

Antibodies against peptide R-6 probably recognize the channel in its native conformation since they are capable of immunoprecipitating the channel in a form that retains high-affinity STX binding. This observation, in turn, implies that the epitope represented by this peptide is accessible on the surface of the channel protein in its mixed micellar form with Lubrol and phospholipid and is not buried in the hydrophobic interior of the native molecule.

We have previously localized the carboxy-terminus of the eel sodium channel to the cytoplasmic surface of the electroplax membrane using antibodies against a synthetic peptide corresponding to amino acids 1781–1792 (Gordon et al., 1987). In channel models that incorporate 4 internal repeat domains sharing similar transmembrane folding patterns (Greenblatt et al., 1985; Guy and Seetharamulu, 1986; Noda et al., 1986a), the location of segment R-6 (AA 927–938 in the stretch between repeat domains 2 and 3) should, therefore, be cytoplasmic as well. In the model proposed by Kosower (1985), however, these repeat domains are not explicitly recognized and amino acids 927–938 are assigned an extracellular location. Our results indicate an intracellular location for this segment of the protein and favor the former folding models over that of Kosower. Since the COOH terminus of the channel protein and the region between domains II and III both face the cytoplasm, each of the 4 homologous repeat domains must contain an even number of transmembrane segments if they share a common folding pattern in the membrane. Whether there are 6 such transmembrane helices as proposed by Noda et al. (1986) or 8 (Greenblatt et al., 1985; Guy and Seetharamulu, 1986) is an issue that remains to be resolved.

Segment R-6 shows little homology between the eel sequence and either of the 2 known rat brain sequences. In these mammalian channels a portion of this segment (residues 936–941) is deleted, and only the first 3 of the remaining amino acids (927–929) are conserved (Noda et al., 1986a). Unlike antibodies raised to highly conserved segments of the channel that cross-react well between species (Y. Li and R. L. Barchi, unpublished observations), antibodies against R-6 do not recognize rat brain or sarcolemmal sodium channels on Western blots and do not react immunocytochemically with these tissues. Thus, it is not surprising that the antibody does not label the neuronal form of the eel channel presumably present in the presynaptic nerve terminal membrane. The absence of labeling of the postsynaptic membrane, however, suggests that the local distribution of the channel in the eel junctional complex may be different from that previously reported in the rat and frog neuromuscular junction (Beam et al., 1985; Caldwell et al., 1986; Haimovich et al., 1987) or that sodium channels in the postsynaptic membrane, if present, may be antigenically distinct from those in the rest of the cell surface.

A recent report, based on the electrophysiological effects of antibodies directed against residues 210–223 of the eel channel, suggests that portions of this putative amphipathic transmembrane helix are accessible from the extracellular surface of the membrane, although ultrastructural localization of antibody

binding was not attempted (Mieri et al., 1987). When considered in conjunction with the localization of the R-6 segment and the carboxy terminus of the sodium channel to the cytoplasmic surface, some constraints can now be placed on the folding models which can be entertained for the protein as a whole. Clearly, much additional work localizing specific aspects of channel structure and relating structure to function is needed before a reliable picture of channel topography can be generated.

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