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Monoclonal Antibodies Raised against Post-translational Domains of the Electroplax Sodium Channel

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Summary. Eleven monoclonal antibodies were identified that recognized eel electroplax sodium channels. All the monoclonal antibodies specifically immunostained the mature TTX-sensitive sodium channel (M_r 265,000) on immunoblots. None of the monoclonal antibodies would precipitate the in vitro translated channel core polypeptide in solution. One monoclonal antibody, 3G4, was found to bind to an epitope involving terminal polysialic acids. Extensive digestion of the channel by the exosialidase, neuraminidase, or partial polysialic acid removal by the endosialidase, endo-*N*-acetylneuraminidase, destroy the 3G4 epitope. 3G4 is, therefore, a highly selective probe for the post-translationally attached polysialic acids. Except for this monoclonal antibody, the epitopes recognized by the remaining antibodies were highly resistant to extensive N-linked deglycosylation. Thus, the monoclonal antibodies may be directed against unique post-translationally produced domains of the electroplax sodium channel, presumably sugar groups that are abundant on this protein (Miller, J.A., Agnew, W.S., Levinson, S.R. 1983. *Biochemistry* **22**:462–470). These monoclonal antibodies should prove useful as tools to study discrete post-translational processing events in sodium channel biosynthesis.

Key Words sodium channel · post-translational domains · monoclonal antibodies · polysialic acid · electric eel

Introduction

The voltage-dependent sodium channel is responsible for the initiation and propagation of the action potential in excitable tissue. To understand better these physical events at the molecular level, this voltage-sensitive membrane protein has been isolated and biochemically characterized from several sources (Miller et al., 1983; Hartshorne & Catterall, 1984; Roberts & Barchi, 1987). Common to all biochemically characterized sodium channels is a large, heavily glycosylated polypeptide with an apparent molecular weight of 260,000, where a significant proportion of the carbohydrate residues are sialic acid.

Glycosylation is essential to the normal biosynthesis and degradation of sodium channels in neuroblastoma cells (Waechter, Schmidt & Catterall, 1983). More recently, it has been reported that enzymatically desialidated channels exhibit functional alterations in voltage-dependent gating and single-channel conductance levels (Schuer et al., 1988; Levinson et al., 1990; Recio-Pinto et al., 1990). Thus, it is of interest to examine these glycosylated domains further to determine the extent of their role in channel biosynthesis and function.

Antibodies specific for such carbohydrate domains might be useful as tools in the study of post-translational sodium channel synthesis. Monoclonal antibodies have been used previously to isolate (Moore et al., 1982; Nakayama, Withy & Raftery, 1982; James, Emerick & Agnew, 1989) and to visualize immunocytochemically (Fritz & Brockes, 1983) electroplax sodium channels. Here we report the characterization of 11 monoclonal antibodies, raised to the eel electroplax sodium channel.

Materials and Methods

Materials

Electric eels were purchased from World Wide Scientific Products. The rabbit reticulocyte lysate system and [^{35}S]methionine (>1100 Ci/mol) were obtained from New England Nuclear. Na^{125}I and ^{14}C-labeled protein standards were purchased from Amersham. Immunoblotting chemicals and materials were purchased from Bio Rad. Neuraminidase, O-glycanase, endoglycosidase-*H*, endoglycosidase-*F* and PNGase-*F* were purchased from Boeringer Mannheim Pharmaceuticals. Endo-*N*-acetylneuraminidase was a gracious gift from Dr. Frederic A. Troy, University of

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California, Davis, CA. All other chemicals were purchased from Sigma.

Radioimmunoassay

A highly purified electroplax sodium channel preparation was iodinated with $Na^{125}I$ using Pierce Iodo-beads as described in the kit. The radiolabeled channels were isolated from the free $Na^{125}I$ using a small, disposable G-50 Sephadex column. Two hundred μl of 1.0% BSA, 0.10% lubrol, 0.23% egg phosphatidylcholine (Sigma, Type V), 0.02% sodium azide and 100 mM sodium phosphate, pH 7.5 was added to RIA tubes containing radiolabeled sodium channel and antibody solution in the desired dilutions. Samples were vortexed and incubated at 4°C for 3 hr, then rabbit anti-mouse or goat anti-rabbit antibodies, coupled to Immunobeads (Bio Rad), were added and incubated at room temperature for 2 hr. The beads were washed 3× in the above buffer and counted for immunoprecipitated radiolabeled channel.

Production of Monoclonal Antibodies

Monoclonal antibodies were raised in Balb-c NS1 mice against purified native or $NaDodSO_4$-denatured sodium channels prepared using the methods described by Miller et al. (1983). Mice were injected with 10 to 25 μg of protein at 14-day intervals until serum samples showed significant anti-sodium channel activity as determined by the above radioimmunoassay. The monoclonal antibody isotypes were determined using the procedure included in the kit from Hyclone. The polyclonal antisera used in this study has been described elsewhere (Thornhill & Levinson, 1987).

Glycosidase Treatment of Vesicular Sodium Channel Preparations

Isolated electroplax sodium channels were reconstituted into asolectin/phosphatidylcholine vesicles following the procedure of Duch and Levinson (1987*a*). Vesiculated sodium channels (1 μg in 9 μl of 50 mM sodium phosphate, pH 6.8) were treated with the following glycosidases: Neuraminidase (*c. perfringens*) at 18.2 U/ml, endo-*N*-acetylneuraminidase at 7.3 U/ml, endoglycosidase-*F* (*F. meningosepticum*) at 17.9 U/ml, PNGase-*F* (*F. meningosepticum*) at 71.4 U/ml, and endoglycosidase-*H* at 0.36 U/ml. Incubations were done at 37°C for 16 hr. The reactions were stopped with the addition of equal volumes of $NaDodSO_4$-sample buffer containing 10% (vol/vol) glycerol, 2% (wt/vol) $NaDodSO_4$, 5% (vol/vol) β-mercaptoethanol, 0.001% (wt/vol) bromophenol blue in 63 mM Tris-HCl, pH 6.8. Samples were heated at 95°C for 2 min and immediately transferred to gels for separation.

Synthesis of Sodium Channel Core Polypeptide

The isolation of electroplax RNAs from electroplax tissue was achieved using the guanidinium thiocyanate method described by Chirgwin et al. (1979). The in vitro translation of channel core polypeptide from isolated electroplax RNAs has been described elsewhere (Thornhill & Levinson, 1987).

Table 1. Physical properties of monoclonal/polyclonal library

Monoclonal antibodies	RIA %cpm recovered[a]	Isotypes	Immunoblot dilutions
1. 2B5	35.7 1:40[b]	IgG_1	1:133
2. 3F6	26.7 1:20	IgG_1	1:133
3. 1F8	13.4 1:20	IgG_1	1:133
4. 3C7	10.3 1:20	IgG_1	1:133
5. 3G4	9.3 1:20	IgM_1	1:67
6. 2A7	7.6 1:20	IgG_1	1:133
7. 1F6	6.7 1:40	IgG_1	1:133
8. 1E7	3.5 1:40	IgG_1	1:133
9. 2C8	2.1 1:20	IgG_1	1:133
10. 1D12	1.7 1:20	IgG_1	1:133
11. 1B7	1.5 1:20	IgG_1	
Polyclonal antibodies			
pAb	61.0 1:20	—	—

[a] The values presented are with background cpm subtracted.
[b] The two dilutions of 1:20 and 1:40 were used for the RIA analysis; the dilution with the highest recovery was noted.

Gel Electrophoresis and Western Blotting

$NaDodSO_4$-polyacrylamide gel electrophoresis was done following the procedure of Laemmli (1970). Western blot transfers were performed according to the procedure of Towbin, Staehelin and Gordon (1979), with the substitution of nylon membranes (Zeta Probe, Bio Rad) for nitrocellulose. The immunoblots were blocked using dry milk (5% wt/vol) in 20 mM HEPES, pH 7.5, and 150 mM NaCl, while antibody incubations were carried out in this solution with 1.0% (wt/vol) dry milk. Antibody dilutions are given in the Table. Molecular weights given for proteins seen on blots were calculated from accompanying ^{14}C-labeled standard proteins.

Abbreviations

PAGE, polyacrylamide gel electrophoresis; $NaDodSO_4$, sodium dodecyl sulfate; HEPES, (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); RIA, radioimmunoassay; cpm, counts per minute; and TTX, tetrodotoxin.

Results

Binding Characteristics of Monoclonal Antibodies Raised against Eel Electroplax Sodium Channel

Our laboratory has raised over 40 hybridoma cell-lines that secrete monoclonal antibodies against sodium channels isolated from the electric organ of the electric eel. Eleven of these clones were chosen for extensive characterization and are listed, with their binding dilutions, in the Table. All 11 monoclonal

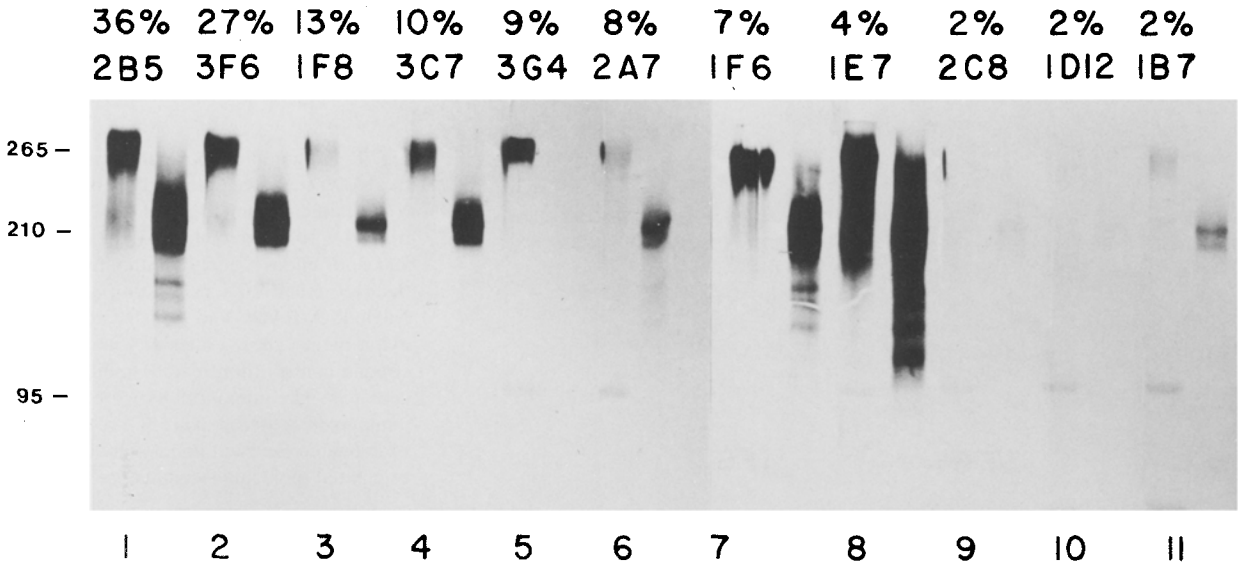

Fig. 1. Immunoblots stained with monoclonal antibodies raised against electroplax sodium channel. Vesiculated sodium channels (1 μg/lane) from electroplax were treated with neuraminidase, separated by 6% $NaDodSO_4$-PAGE and electrophoretically transferred to nylon membranes. Each strip contains two lanes, control and neuraminidase-treated samples. Strips 1-11 were immunostained with the designated monoclonal antibodies. All the antibody incubations were done at 1 : 133 dilutions except 3G4, which was at 1 : 67. Lanes *1*, *2* and *7* were developed for 15 min, lane *8* was developed for 4 min, and the remaining lanes were developed for 1 hr. 1E7 was knowingly over developed, even at 4-min development time, to demonstrate its high immunostaining ability. This is intriguing since its RIA recovery is very low. RIA values for each of the antibodies are shown above the corresponding strips. Representative molecular weight values are depicted to the left of the immoblots. (The light band seen at 95K is a colored standard added to the control lanes to facilitate strip slicing.)

antibodies immunoprecipitate ^{125}I-labeled sodium channel in RIAs, with precipitated recoveries ranging from 2 to 36% of the total cpm. Moreover, all the monoclonal antibodies used in this study stained the mature TTX-binding component on immunoblots (*see* Fig. 1).

The eel electroplax sodium channel has been shown by Miller et al. (1983) to be heavily glycosylated, with carbohydrate moieties comprising about 30% of the mass of the molecule. Of this, approximately 40% of the carbohydrate is sialic acid. Thus we were first prompted to screen our monoclonal library for antibodies that would show some specificity for the channel sialic acids. Vesiculated sodium channels were enzymatically treated with neuraminidase, an exosialidase (Meindl et al., 1974), and then analyzed by $NaDodSO_4$-PAGE and immunoblot. Removal of the sialic acids caused a reduction of 55K in the apparent molecular weight of the channel peptide, as seen on a 6% mini gel. Eight of the monoclonal antibodies specifically immunostained the mature and treated channels (M_r 265,000 and 210,000; Fig. 1). While not visible in photoreproduction of the original blot, antibodies 2C8 and 1D12 very faintly stained untreated channel protein, with a stronger (yet still weak) reaction to neuraminidase-treated protein (lanes *9* and *10*). Only one monoclonal antibody, 3G4, did not react with the channel after the neuraminidase treatment (strip 5; Fig. 1). This was the only monoclonal antibody whose binding was dependent on the presence of sialic acid. Five of the antibodies (2A7, 1B7, 2C8, 1D12 and 1F8) immunostained the treated channel (M_r 210,000) more intensely than the untreated channel; possibly their epitopes are partially blanketed by the channel sialic acids. None of the monoclonal antibodies immunostained a discrete band in the diffusely staining band at 265K. Also, high immunostaining intensities, seen on our immunoblots, were not necessarily associated with high RIA recoveries. For example, both 1F6 and 1E7 exhibited intensely immunostaining profiles, but had very low RIA recoveries.

3G4 Is Specific for Channel Polysialic Acids

The eel electroplax sodium channel has been shown to contain poly-α-2,8-sialosyl carbohydrate units (James & Agnew, 1987). To determine if the 3G4 epitope required this form of sialic acid, the electroplax channel was treated with neuraminidase, as above, and with endo-*N*-acetylneuraminidase (endo-*N*). Endo-*N* is specific for polysialosyl chains,

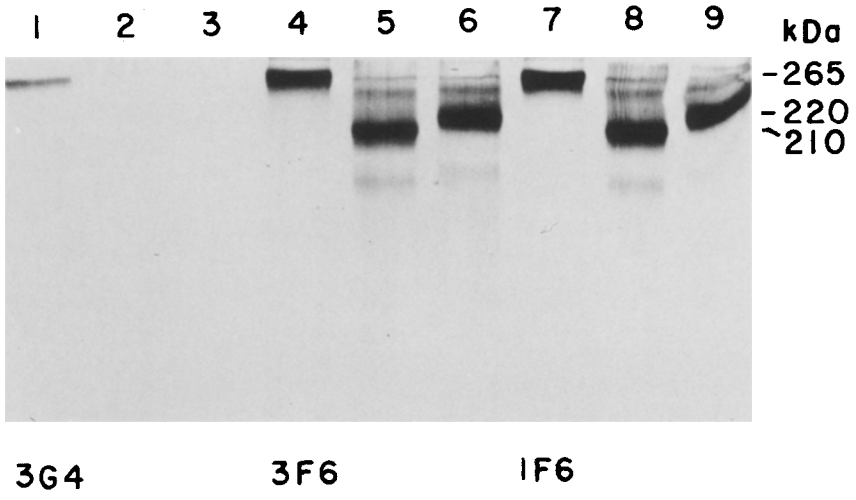

Fig. 2. Immunoblots of neuraminidase-treated vesiculated sodium channel. Vesiculated sodium channel was treated with neuraminidase (lanes *2*, *5* and *8*) or endo-*N*-acetylneuraminidase (lanes *3*, *6* and *9*), separated on a 6% $NaDodSO_4$-PAGE and transferred to nylon membranes. Lanes *1*, *4* and *7* contain control (nontreated) sodium channels. The immunoblots were immunostained with 3G4, 3F6 or 1F6, as indicated for each panel. The calculated molecular weights are noted

requiring a substrate with a minimum of five sialyl residues (Hallenbeck et al., 1987). Also, endo-*N* may randomly cleave a polysialosyl chain, ultimately leaving a short oligomer of sialic acid (DP4) attached to the remaining sugar. Immunoblots containing the neuraminidase-treated channels were challenged with the monoclonal antibodies 3G4, 1F6 and 3F6 (Fig. 2). 1F6 and 3F6 were used as control antibodies to monitor the removal of the sialic acids. Both the neuraminidase- and endo-*N*-treated channels were immunostained by 1F6 and 3F6; however, 3G4 only recognized the nontreated channels. Since endo-*N* does not remove the four innermost sialosyl residues nearest the polypeptide backbone, it appears that 3G4 recognizes an epitope that requires the sialic acids located in the more terminal regions of the polysialosyl chains.

The Monoclonal Antibodies Do Not Precipitate the in vitro Channel Core Polypeptide

Since the neuraminidase treatment inhibited the interaction of only one monoclonal antibody (3G4), we next examined the ability of these antibodies to interact with the unmodified channel polypeptide itself. To do this, the electroplax sodium channel core polypeptide was translated in vitro using a rabbit reticulocyte translation system in the presence of [^{35}S]methionine. RNAs isolated from electroplax tissue, were added as previously described (Thornhill & Levinson, 1987). When immunoprecipitated using a polyclonal antibody, the apparent molecular weight of the translated core polypeptide, on a 5–30% $NaDodSO_4$-PAGE linear gradient gel, was 230,000 (pAb, Fig. 3). The precipitation of this core polypeptide by the polyclonal antibodies was completely blocked by mature eel sodium channel. None

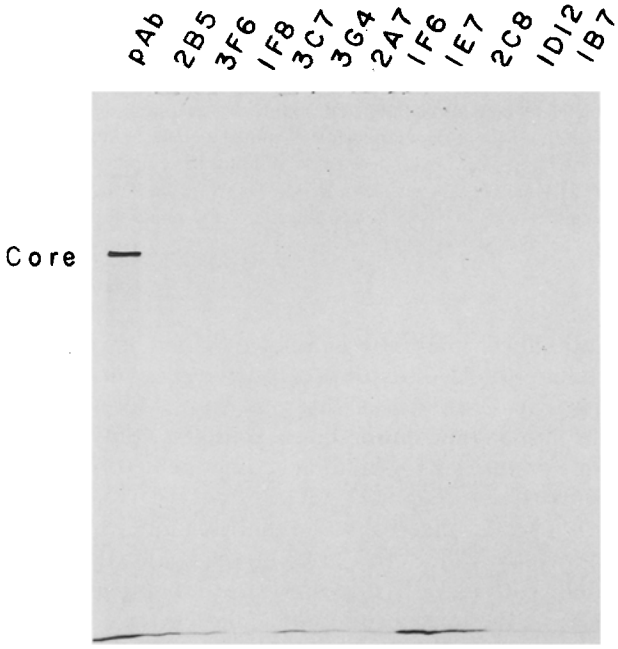

Fig. 3. Autoradiogram of the [^{35}S]methionine-labeled sodium channel core polypeptide, translated in vitro from electroplax total RNAs and precipitated from solution with the noted antibodies. Only the polyclonal antibodies (pAb, lane 1) precipitated the nonglycosylated channel protein in solution. None of the 11 monoclonal antibodies would immunoprecipitate the core polypeptide. The precipitated polypeptide was separated on a 5–30% linear gradient $NaDodSO_4$-PAGE

of the monoclonal antibodies precipitated this core polypeptide from solution (Fig. 3). Control experiments were done to ensure that under the conditions used the monoclonal antibodies would precipitate the mature sodium channel from solution. This result suggests that post-translational processing of the protein is necessary for the development of these monoclonal epitopes.

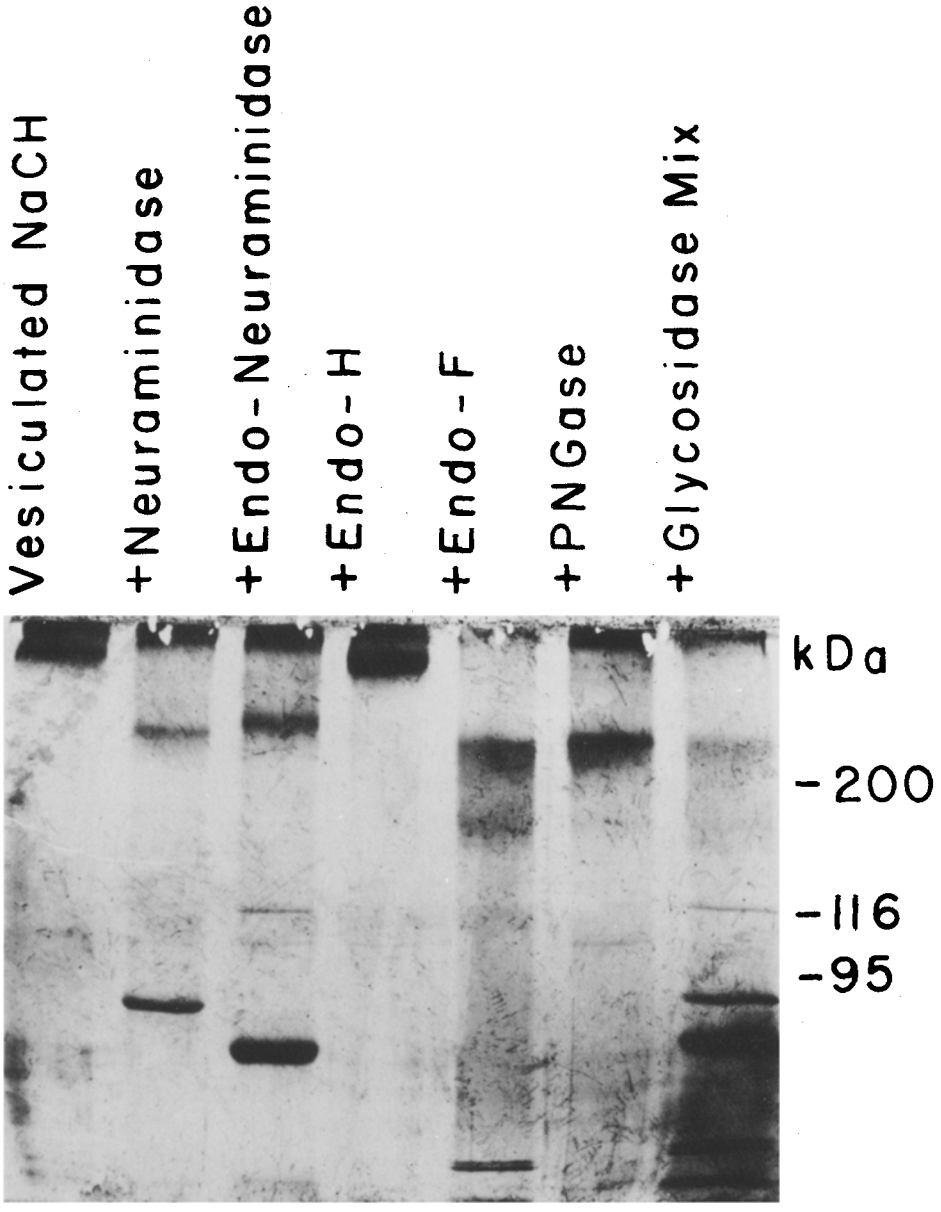

Fig. 4. Silver-stained gel profiles of glycosidase-treated vesiculated sodium channel. The gel contains control (nontreated) channel (lane 1); channel treated with neuraminidase (lane 2); endo *N*-neuraminidase (lane 3); endoglycosidase-*H* (lane 4); endoglycosidase-*F* (lane 5); PNGase-*F* (lane 7); and channel treated with a mixture of all the above glycosidases. The prominent lower molecular weight proteins seen in lanes 2–6 are the added glycosidases; all the glycosidases are present in lane 7. Standard protein molecular weights are given on the side

Antibody Epitopes Are Resistant to Glycosidase Treatments

To determine whether the monoclonal antibodies were to N-linked oligosaccharides on the channel, vesiculated sodium channels were treated with the endoglycosidases -*H*, -*F*, and PNGase-*F* to remove branched N-linked oligosaccharides (Fig. 4). Only endo-*F* and PNGase-*F* (lanes 5 and 6) caused a shift in the apparent molecular size of the peptide; endo-*H* was without discernible effect (lane 4). Apparently, channels in these vesicles were largely right-side out, as little staining remained at 265K after the glycosidase treatments. Similar asymmetry of channel orientation using this system has been previously described (Duch & Levinson, 1987*b*). To remove as much of the carbohydrate as possible, neuraminidase, endo-*N*, and the three endoglycosidases mentioned above were mixed and used to treat the vesiculated channels. After this treatment, the M_r shift

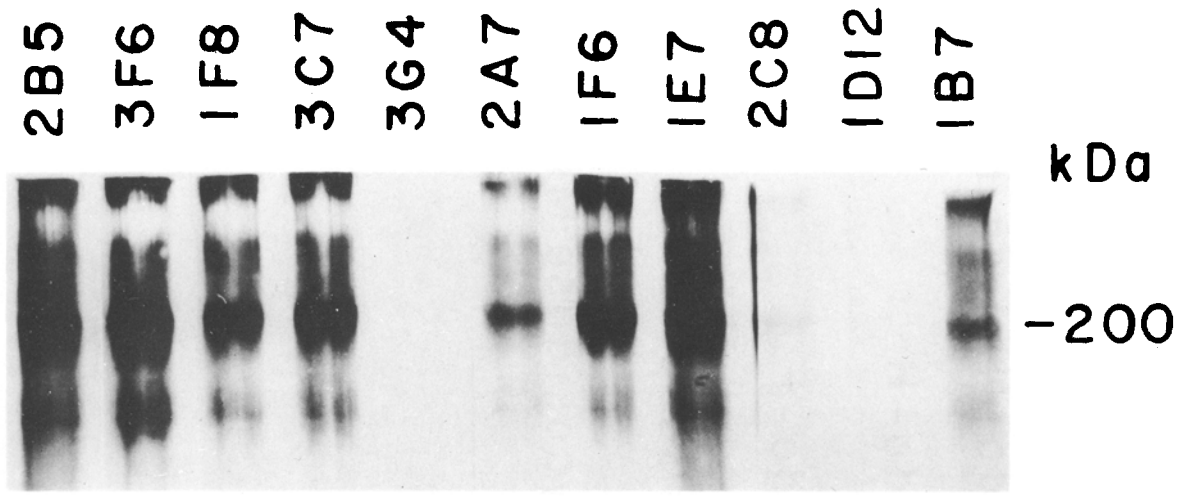

Fig. 5. Immunoblots of extensively deglycosylated sodium channels seen in lane 7, Fig. 4. All of the monoclonal antibodies recognize the channel after the glycosidase treatment except 3G4, which is specific for polysialic acids. Again, strips stained with 2C8 and 1D12 photoreproduced poorly but do, nonetheless, stain the treated channel

was similar to that of endo *F*-treatment alone (M_r of 210,000). When blots containing these extensively deglycosylated channels (as seen in lane 7; Fig. 4) were challenged with our monoclonal library, all antibodies, except 3G4, immunostained the treated channel (Fig. 5). 2C8 and 1D12's results photographed poorly, but the 210 kDa channel protein was present on the stained strips nonetheless.

In an attempt to determine whether O-glycosidic linkages existed, channel protein was subjected to mild alkaline treatment (β-elimination reaction) to remove specifically O-linked oligosaccharides (Montreuil et al., 1986). After treatment, the channels appeared to aggregate and would not run into the separating phase during PAGE. Moreover, O-glycanase, a glycosidase specific for O-linked oligosaccharides, lacked effect on either the electrophoretic mobility of the protein or monoclonal antibody staining on immunoblots (*data not shown*).

Discussion

The biosynthesis of the electroplax sodium channel in electrocytes and oocytes has been partially described in previous studies using polyclonal antibodies (Thornhill & Levinson, 1987). In these studies, numerous bands on $NaDodSO_4$-PAGE were detected after metabolic labeling with [^{35}S]methionine. Pulse-chase techniques were used to determine the sequence of $NaDodSO_4$-PAGE mobility changes that reflected underlying post-translational processing events. In the present studies, monoclonal antibodies were investigated for their binding abilities as a future means of following sodium channel biosynthesis. We find that certain of these monoclonals recognize epitopes that depend on specific post-translational processes and, therefore, will be useful in further studies of the biosynthetic history of the channel.

Carbohydrate moieties are strongly implicated in the composition of these epitopes for several reasons. First, the electroplax sodium channel is highly glycosylated, and the high degree of antigenicity of carbohydrate, coupled with the expectation that it could sterically mask a large part of the underlying protein core, suggests that sugar-directed antibodies would predominate in a monoclonal library. More directly, none of these monoclonals immunoprecipitated the unglycosylated channel protein core as synthesized via in vitro translation, but do precipitate the fully processed mature channel under similar conditions. The chemical nature of the epitopes themselves is uncertain. While it is likely that most of the antibodies will bind directly to sugar groups, it is also possible that sugar groups are indirectly involved in the folding or maintenance of conformationally dependent epitopes on the peptide core that are absent in the in vitro synthesized peptide. Such sites, however, would have to be highly stable to harsh denaturing conditions, since these antibodies were raised against, and recognize, the reduced, $NaDodSO_4$-denatured protein.

Except for the sialic acid-dependent antibody, 3G4, the monoclonal antibodies in our library appear to recognize a highly glycosidase-resistant site, as treatment with a variety of N-linked oligosaccharide

or sialic acid-directed enzymes failed to destroy antigenicity. It seems likely that significant carbohydrate remained linked to the channel after these treatments, since the electrophoretic microheterogeneity characteristic of the mature channel was still observed, although reduced (Fig. 4).

Horse IgM antibodies, raised to *N. memningitidis* bacterial poly-α-2,8-sialosyl carbohydrate units, have been used to isolate the sodium channel from solubilized electroplax membranes (James et al., 1989). Only one of the 11 antibodies studied here (3G4) was specific for polysialic acids on the channel and was the only monoclonal antibody in our library to be of the IgM isotype. This IgM monoclonal antibody represents the first probe raised against the electroplax sodium channel's polysialic domain. It will serve as a highly specific probe in future studies involving the polysialic acids and their role in the electroplax sodium channel structure and function.

1E7 specifically precipitates the sodium channel from solubilized electroplax membranes, as detected on Western blots (*data not shown*). However, when combined, none of the 11 monoclonal antibodies precipitate immunodetectable amounts of sodium channels from the solubilized membranes of eel brain, heart, or muscle. Since the electroplax is embryologically derived from muscle tissue, it is surprising that the eel muscle sodium channels do not express an epitope similar to the electroplax channel. In addition to these studies, our monoclonal library does not immunostain blots containing proteins from rat heart, rat brain or *Drosophila* brain. These results are consistent with previous studies (Fritz & Brockes, 1983; Fritz et al., 1983) reporting that electroplax sodium channel-specific monoclonal antibodies were unreactive to rat, frog or chick brain sodium channels. We suggest that these immunological differences arise from discrete post-translational domains that are likely to be highly species and tissue specific than from differences in sodium channel polypeptide sequences, which tend to be conserved over diverse phyla (Noda et al., 1984; Salkoff et al., 1987; Kayano et al., 1988).

The high degree and apparent heterogeneity of glycosylation seen in the sodium channel dictates a complex post-translational pathway. To unravel this complex biochemical progression, specific probes are necessary for the observation of channel biosynthesis, from its translational nascence, through its processing in intracellular organelles, to its functional insertion into the plasma membrane. The post-translationally specific monoclonal antibodies described here represent a possible approach to the production and design of such probes.

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