Characterization of Saccharide Moiety in the Electroplax Sodium Channel

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Carbohydrate chains on the large peptide of the voltage-sensitive sodium channel from *Electrophorus electricus* electroplax have been partially characterized by the lectin-blotting technique combined with digestion using three glucosidases: neuraminidase, *endo*-β-*N*-acetylglucosaminidase H, and peptide: *N*-glycosidase F. The results show that both N-linked oligosaccharides and O-linked (mucin-type) oligosaccharides are present. In N-linked oligosaccharides, the results suggest the presence of complex- and hybrid-type oligosaccharides which contain bisecting *N*-acetylglucosamine(s), as well as the complex-type oligosaccharides with the α-Fuc-GlcNAc-(Asn) residue(s). In O-linked oligosaccharides, they must carry Galβ1→3GalNAc- moieties which contain NeuNAc residues in the terminal.

Keywords oligosaccharide chain; sodium channel; lectin-blot; glycosidase; *N*-glycoside; *O*-glycoside

Introduction

Voltage-sensitive sodium channels that are responsible for the depolarizing currents of the action potential have been isolated from several sources including eel electroplax, rat and rabbit muscle and rat brain (for review, see ref. 2). Successive efforts following the first elucidation of the primary structure of electroplax sodium channel[3)] have made it possible to clone and sequence the sodium channels from some other origins.[2)] The functionally important component of each channel species is a large polypeptide (M_r 250—300 kDa). It is a striking characteristic that all of the large sodium channel peptides so far studied are heavily glycosylated.[2)] This large peptide (250 kDa) is the sole component of the electroplax sodium channel to express its entire function, and contains approximately 29% carbohydrate by mass.[4)] Its sugar composition,[4)] the existence of α-2,8-linked polysialic acids,[5)] and sensitivity to several glycosidases[5)] have been reported. However, no further structural characterisation of the carbohydrate moiety has been performed. We therefore undertook to collect information about carbohydrate moieties as the first step in elucidating their structures. The carbohydrate moieties are examined by lectin-blotting technique[6)] with five lectins of different specificities after successive digestion with three glycosidases of the electroplax sodium channel protein. In this paper, we present partial characterization of the carbohydrate chains of the electroplax sodium channel.

Results and Discussion

Carbohydrate Composition of the Eel Electroplax Sodium Channel The purified sodium channel protein shows a broad but single band at 250 kDa (Fig. 1A) owing to heavy glycosylation of the polypeptide portion which is homo-

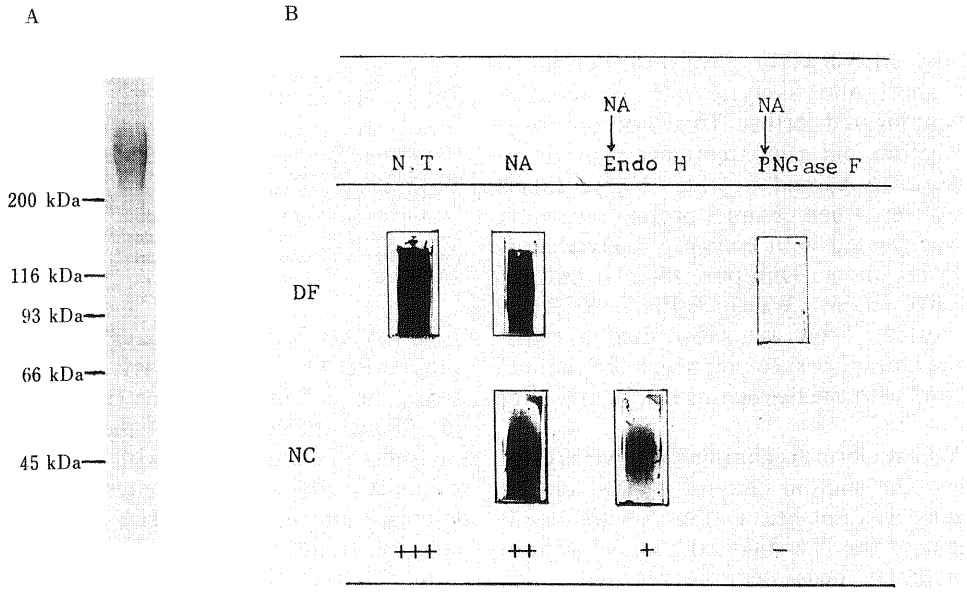

Fig. 1. Lectin Blotting of the Sodium Channel with HRP-Conjugated WGA before and after Glycosidase Treatments

(A) The purified eel electroplax sodium channel was analyzed by SDS-PAGE on a 6% polyacrylamide gel and silver-stained. Only a single protein band was observed at *ca.* 250 kDa. The sample was used for the subsequent experiments. (B) The purified sodium channel on SDS-polyacrylamide gel was electrophoretically transferred to Durapore filter (DF) or nitrocellulose (NC) sheets. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated WGA before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase; Endo H, *endo*-β-*N*-acetylglucosaminidase H; PNGase F, peptide: *N*-glycosidase F) on the sheets. Only the regions of the lectin-blotted sheets at molecular mass higher than 200 kDa are shown. Symbols +++, ++, +, and − show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

TABLE I. Carbohydrate Composition of the Purified Sodium Channel from *E. electricus*

	Carbohydrate/sodium channel protein (mol/mol)[a]
Glucosamine	17[b]
Galactosamine	4[b]
Mannose	29[c]
Fucose	6[c]
Galactose	24[c]
Xylose	34[c]
N-Acetylneuraminic acid	92[d]

a) Moles of the purified sodium channel protein were determined by amino acid analysis and calculated as 95 mol for lysine residues based on the reported value from the total primary structure.[3] *b*) Determined simultaneously by amino acid analysis. *c*) Determined by neutral carbohydrate analysis as described in Experimental. *d*) Determined by sialic acid analysis as described in Experimental.

geneous.[3] Carbohydrate composition of the purified channel protein was determined in terms of molar ratio to the sodium channel protein, based on the molecular mass of 250 kDa (Table I). The molecular mass of the total carbohydrates was calculated as 45 kDa, which is in good accordance with the expected value obtained by subtraction of protein mass (208321 Da calculated based on the primary structure[3]) from the molecular mass of the intact sodium channel (250 kDa) estimated by sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 1A). *N*-Acetylneuraminic acid (92 mol/mol of channel) is the most abundant component, as has been indicated in the literature.[4,5] In addition to glucosamine, galactosamine was determined for the first time to be component in the electroplax sodium channel. Taken together with the determination of a considerable amount of galactose residues, the presence of O-linked saccharides (mucin-type) was indicated in the channel molecule.

Lectin-Blot Assay of Oligosaccharide Chains in the Electroplax Sodium Channel before and after Treatment with Glycosidases The electroplax sodium channel protein was purified by SDS-PAGE, electrotransferred to a Durapore fllter sheet and assayed with horseradish peroxidase (HRP)-conjugated lectins. This procedure was used for the assay before and after treatment with glycosidases, neuraminidase (NA) and peptide: *N*-glycosidase F (PNGase F). However, when channel protein (eggwhite albumin as well) was treated with *endo-β-N*-acetylglucosaminidase H (Endo H) on the Durapore filter, it did not react with any of five lectins (WGA, E-PHA, RCA_{120}, PNA, and LCA) tested. Then, we substituted a nitrocellulose sheet for the Durapore sheet, on which the channel protein could be tested with the five lectins before and after Endo H treatment.

(i) Assay with Wheat Germ Agglutinin (WGA): WGA–HRP heavily stained the sodium channel blotted on the sheet, but the staining was not heavy after NA treatment (Fig. 1B). Treatmemt of the NA-digested channel protein with Endo H remarkably decreased the reactivity with WGA. Furthermore, upon PNGase F digestion, the NA-digested channel protein lost its WGA reactivity completely. These results suggest that the electroplax sodium channel protein bears NeuNAc and bisecting GlcNAc moieties in both hybrid-type *N*-glycans and complex-type N-glycans.

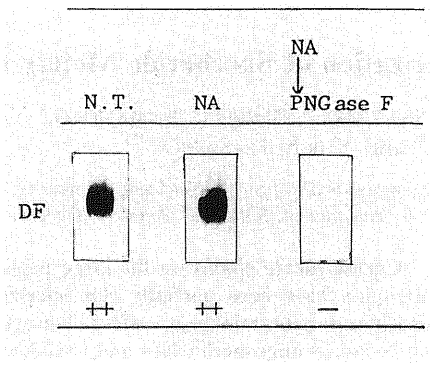

Fig. 2. Detection of Oligosaccharide Chains of the Sodium Channel with HRP-Conjugated E-PHA

The purified sodium channel on SDS-polyacrylamide gel was electrophoretically transferred to Durapore filter (DF) sheets. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated E-PHA before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase; PNGase F, peptide: *N*-glycosidase F) on the sheets. Symbols + + and — show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

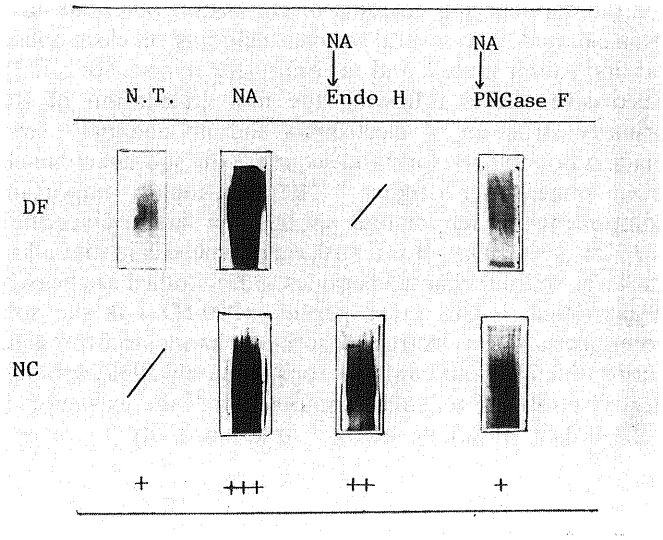

Fig. 3. Detection of Oligosaccharide Chains of the Sodium Channel with HRP-Conjugated RCA_{120}

The purified sodium channel was transferred to Durapore filter (DF) or nitrocellulose (NC) sheets by Western blotting. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated RCA_{120} before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase; Endo H, *endo-β-N*-acetylglucosaminidase H; PNGase F, peptide: *N*-glycosidase F) on the sheets. Symbols + + +, + +, and + show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

(ii) Assay with Erythroagglutinating Phytohemagglutinin (E-PHA): E-PHA–HRP stained the channel protein clearly on a Durapore filter (Fig. 2). NA treatment did not change the reactivity of the protein with E-PHA and the subsequent digestion with PNGase F abolished the reactivity to the lectin. These results support the suggestion described above, since E-PHA has specificity for biantennary and triantennary complex-type *N*-glycans.[7–9]

(iii) Assay with *Ricinus communis* $Agglutinin_{120}$ (RCA_{120}): RCA_{120} can specifically bind both galactose residue at the nonreductive terminal and Galβ1→4GlcNAc moiety in complex- and hybrid-type *N*-glycans strongly, and Galβ1→3GalNAc moiety in mucin-type oligosaccharides moderately.[10–12] RCA_{120}–HRP stained the intact channel protein only slightly, but the protein was

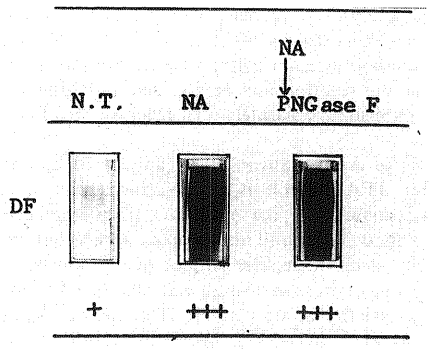

Fig. 4. Reaction of HRP-Conjugated PNA with the Sodium Channel before and after Treatment with Glycosidases

The purified sodium channel was transferred to Durapore filter (DF) sheets by Western blotting. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated PNA before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase; PNGase F, peptide: *N*-glycosidase F) on the sheets. Symbols + + + and + show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

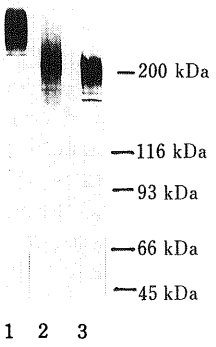

Fig. 5. SDS-PAGE Analysis of the Electroplax Sodium Channel before and after Treatment of Glycosidases

The purified sodium channel was treated in the absence (lane 1) and presence of neuraminidase (lane 2) or neuraminidase followed by PNGase F (lane 3). After treatment, the samples were boiled for 3 min in the 50 mM dithiothreitol, subjected to SDS-PAGE on a 6% polyacrylamide gel, and stained with silver.

heavily stained after NA treatment (Fig. 3). This is due to the exposure of terminal galactose residue caused by removal of sialic acid residues. After Endo H digestion, the NA-treated protein was weakly stained by RCA_{120}–HRP stain on nitrocellulose sheets, the presence of hybrid-type *N*-glycans containing Galβ1→4GlcNAc moieties being indicated. PNGase F digestion significantly reduced the reactivity of the NA-treated protein to RCA_{120}–HRP stain indicating the presence of complex-type *N*-glycans. The fact that NA and PNGase F-treated protein retains the reactivity to RCA_{120} suggests strongly that the Galβ1→3GalNAc moiety exists in the portion of mucin-type oligosaccharides which is resistant to the PNGase F digestion of the electroplax sodium channel protein.

(iv) Assay with Peanut Agglutinin (PNA): PNA recognizes Galβ1→3GalNAc moieties in mucin-type oligosaccharides strongly, and Galβ1→4GlcNAc moieties in complex-and hybrid-type *N*-glycans moderately.[10] This is rather the inverse of the specificity of RCA_{120}. NA treatment markedly enhanced the reactivity of the channel protein to PNA as is well shown in (iii). However, the behavior of the protein at the PNGase F digestion was different: it retained the reactivity to the lectin (Fig. 4). These are complementary results obtained with RCA_{120}. Since PNGase F removes all the N-glycosidic moieties in the NA-treated channel protein, as shown by the insensitivity to WGA, E-PHA, and LCA (see section v), it is deduced that the Galβ1→3GalNAc moiety exists in the portion of mucin-type oligosaccharides.

The observation that molecular size lowered to *ca.* 200 kDa by NA- and PNGase F-treatments on SDS-PAGE (Fig. 5), which is in good accordance with the molecular mass of channel protein (208321 Da) estimated by the primary structure, also supports that PNGase F effectively cleaves N-linked oligosaccharides of the NA-treated sodium channel. Even after such extensive removal of *N*-glycans, some residual microheterogeneity observed as a rather broad band for the channel protein of *ca.* 200 kDa (lane 3 in Fig. 5), is another evidence for the existence of *O*-glycans. The primary amino acid sequence includes 10 potential *N*-glycosylation sites,[3] 9 of which may be extracellular and 7 of which are conserved in two brain

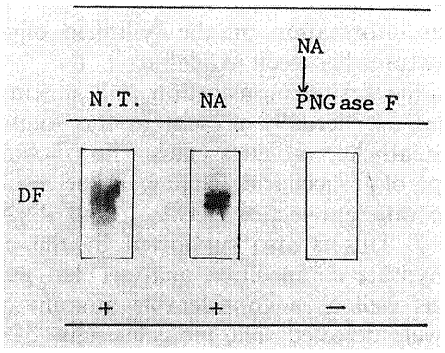

Fig. 6. Reaction of HRP-Conjugated LCA with the Sodium Channel before and after Treatment with Glycosidases

The purified sodium channel protein was transferred to Durapore filter (DF) sheets by Western blotting. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated LCA before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase; PNGase F, peptide: *N*-glycosidase F) on the sheets. Symbols + and − show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

polypeptides that have been cloned.[13] Remarkable reduction in the apparent molecular mass of the channel protein (from 250 kDa to *ca.* 200 kDa) by removing *N*-glycans suggests that N-linked oligosaccharides are major carbohydrate constituents in the electroplax sodium channel protein: grossly *ca.* 24 saccharide residues per one of the 10 potential *N*-glycosylation sites by assuming 210 as average molecular weight of saccharide unit. The number of O-linked saccharide units may be less than 4, the determined galactosamine residues (Table I).

(v) Assay with *Lens culinaris* Agglutinin (LCA): LCA–HRP stained both intact and NA-treated sodium channel protein lightly; the extent of staining in the two being similar. The NA-treated protein lost the reactivity to LCA upon PNGase F digestion (Fig. 6). suggesting the presence of a complex-type oligosaccharide bearing the α-Fuc→GlcNAc-(Asn) residue.

Oligosaccharide Chains in the Electroplax Sodium Channel In the present work, we have characterized the gross structures of several oligosaccharide chains in the electroplax sodium channel from *Electrophorus electricus* by the lectin-blotting technique combined with digestion using glycosidases of different substrate specificity. This technique was first developed by Kijimoto-Ochiai *et al.*[6] and

proved feasible for detection of oligosaccharide moieties *in situ* using several glycoproteins of known oligosaccharide structure, although there are some limitations; for example, detection is qualitative rather than quantitative, and broad specificity and cross-reactivity of lectins sometimes hamper elucidation of the exact structure of oligosaccharide chains.[6] However, the present work shows that the technique affords information reasonable and consistent with chemical compositional analysis and new information as well. The results strongly suggest that not only N-linked oligosaccharides but also O-linked (mucin-type) oligosaccharides are present in the sodium channel protein, although the former are more adundant.

As N-linked oligosaccharides, they suggest the presence of complex- and hybrid-type oligosaccharides which contain bisecting *N*-acetylglucosamine, as well as complex-type oligosaccharides with the α-Fuc→GlcNAc-(Asn) residue. Although the presence of hybridtype *N*-glycans was suggested previously in the electroplax sodium channel,[5] no further information on the N-linked oligosaccharide chain structures has been available.

This is the first demonstration that mucin-type oligosaccharides are actually present in the sodium channel. Galβ1→3GalNAc moieties must be included in the mucin-type oligosaccharide chains, as the results obtained using two different lectins, RCA_{120} and PNA, are complementary. This is also supported by the present data of carbohydrate composition analysis that galactosamine residues as well as a considerable amount of galactose residues were detected. The molcular mass of the channel protein (250 kDa) was reduced to *ca.* 210 kDa by NA digestion, while further PNGase F treatment caused decrease in its molecular mass to 200 kDa, only by 10 kDa, demonstrating that sialic acid is apparently the major component of the carbohydrate composition as observed previously.[4,5,14-16] α-2,8-Linked sialic acid homopolymers which are uncommon in vertebrate cells were characterized in the electroplax sodium channel.[5] Sialic acids may contribute to surface charge effects on ion conductance and neurotoxin interactions.[17] As sialic acid residues can be remoed efficiently by the NA-treatment as shown here, it will be intriguing to assess their functional significance in the sodium channel.

Experimental

Materials Lectins (WGA, E-PHA (*Phaseolus vulgaris* agglutinin), RCA_{120}, PNA, LCA) conjugated with HRP were purchased from Honen Oil Company. NA from *Achromobater ureafacints* was purchased from Nakarai Chemical Ltd. Endo H from *Streptomyces griseus* and PNGase F from *Flavobacterium meningosepticum* were obtained from Seikagaku Kogyo Company Ltd. and Boehringer Mannheim, respectively. Durapore and nitrocellulose sheets were obtained from Millipore. Other chemicals (reagent grade) were obtained commercially.

Isolation of the Sodium Channel Protein from Electroplax Lubrol PX extracts from *Electrophorus electricus* electroplax was further purified by successive chromatography of DEAE-Sephadex, Sepharose 6B, Sepharose 4B, and TSK G4000SW, as described previously.[3]

Carbohydrate Composition Analysis The purified sodium channel proteins (100 pmol) were dissolved in 100 μl of 0.1 M ammonium bicarbonate and subjected to composition analysis as follows:

1. Analysis of Neutral Carbohydrates: To ten microliters of the sample was added 1 N trifluoroacetic acid (TFA, 100 μl) and the resultant solution was sealed in a Pyrex tube *in vacuo*, followed by hydrolysis at 110 °C. After 6 h, the acid solvent was evaporated to dryness and residual materials were dissolved into water (50 μl) and subjected to analysis. Carbohydrate analyses were performed by HPLC using an anion exchange column (Shimadzu ISA-07/S2504, 4 × 250 mm) and determined as post-column method by colorimetry after reaction with thiourea. Detailed procedures for the reaction and HPLC determination were described[18] and will be published separately (T. Hachisu, S. Tsunasawa, and F. Sakiyama).

2. Analysis of Amino Sugars: An aliquot of the aqueous solution obtained after TFA hydrolysis as described in 1, was reacted with phenylisothiocyanate and the resultant phenylthiocarbamoyl derivatives[19,20] of amino sugars and amino acids were determined by Pico-Tag method. Briefly, a ten microliter aliquot of the aqueous solution (50 μl) was evaporated to dryness in a small vial tube (5 × 40 mm) and a mixture of EtOH : H_2O : triethylamine (2 : 2 : 1, 20 μl) was added. After evaporation of the solvent to dryness *in vacuo*, the residue was reacted with a mixed solution (20 μl) of phenylisothiocyanate : EtOH : H_2O : triethylamine (0.2 : 79.8 : 10 : 10) for 30 min at 50 °C. The solvent was removed by evaporation, followed by quantitative analysis by the Pico-Tag method.[19] Phenylthiocarbamoyl derivatives of galactosamine and glucosamine were eluted at 4.5 and 5 min, respectively.

3. Determination of Sialic Acid: A ten microliter aliquot of the purified sodium channel protein was dried under reduced pressure and hydrolyzed with 0.025 N sulfuric acid (100 μl) for 1 h at 80 °C in a sealed tube. The hydrolysate was directly analyzed by HPLC using a μBondasphere NH_2-column (5 μm, 100 Å, 3.9 × 150 mm, Waters), 3 mM KH_2PO_4–70% (v/v) acetonitrile as solvent, flow rate at 1.0 ml/min, and monitored at 205 nm. *N*-Acetylneuraminic acid was eluted at 18 min under these conditions.

SDS-PAGE and Electrotransfer Sodium channel proteins were subjected to electrophoresis on SDS-PAGE (6% acrylamide gels) according to Laemmli.[21] Proteins on the gel were transferred to either Durapore or nitrocellulose sheets in 25 mM Tris/192 mM glycine/20% (v/v) methanol[22] at 70 V for 24 h at 4 °C. Either the Durapore or the nitrocellulose paper with blotted protein was washed in Tris–Tween–saline (10 mM Tris–HCl, pH 7.5/0.05% Tween 20/0.15 M NaCl) and reacted with HRP-conjugated lectins as follows.

Lectin Blotting This method is originally developed by Kijimoto-Ochiai *et al.*[6] Lectins conjugated with HRP were diluted appropriately with Tris–Tween–saline to get a suitable color development. HRP–WGA, HRP–E–PHA, HRP–RCA_{120}, HRP–PNA, and HRP–LCA were used with 272 milli-purpurogallin units/0.7 μg/ml, 128 milli-purpurogallin units/1.3 μg/ml, 100 milli-purpurogallin units/1.3 μg/ml, 1770 milli-purpurogallin units/19 μg/ml, and 664 milli-purpurogallin units/7.5 μg/ml, respectively. After reacting with an HRP-conjugated lectin for 1 h, the sheet was washed for 1 h in Tris–Tween–saline with 4 changes and then reacted with 0.03% 3,3′-diaminobenzidine in 15 mM sodium phosphate buffer, pH 5.0, containing 6×10^{-3}% H_2O_2 at room temperature until the color developed (usually 1—5 min), as described in the literature.[6]

Glycosidase Digestion A band corresponding to the sodium channel (250 kDa) transferred to either Durapore or nitrocellulose sheet was cut out. The strip of the sheet was digested with each glycosidase as follows: Neuraminidase, with 0.1 U/ml of the enzyme for 2 h at 37 °C in 50 mM sodium phosphate/25 mM sodium citrate (pH 5.5) containing 0.05% SDS; Endo H, with 80 mU/ml of the enzyme for 15 h at 37 °C in 50 mM sodium phosphate/25 mM sodium citrate (pH 5.0) containing 0.05% SDS; peptide: *N*-glycosidase F, with 0.5 U/ml of the enzyme for 15 h at 37 °C in 0.5 M sodium phosphate (pH 7.5) containing 0.1% SDS/1% Triton X-100. The enzyme-treated strip was washed with 10 mM Tris (pH 7.5)/0.05% Tween-20/0.15 M NaCl for 40 min with 4 changes, followed by reaction with HRP–lectins as described above.

Acknowledgements This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan, and a grant (to H. N.) from the Fugaku Trust for Medical Research.

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